

Development of an Optical Tissue Clearing Laser Probe System

Changmin Yeo¹, Heesung Kang², Yunjin Bae², Jihoon Park², J. Stuart Nelson³,
Kyoung-Joung Lee^{2,4}, and Byungjo Jung^{2,4*}

¹Training Management Team, Medical Device Information & Technology Assistance Center,
Seoul 152-718, Korea

²Department of Biomedical Engineering, Yonsei University, Wonju 220-710, Korea

³Beckman Laser Institute, University of California, Irvine, CA 92617, U.S.A

⁴Institute of Medical Engineering Yonsei University, Wonju 220-710, Korea

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Although low-level laser therapy (LLLT) has been a valuable therapeutic technology in the clinic, its efficacy may be reduced in deep tissue layers due to strong light scattering which limits the photon density. In order to enhance the photon density in deep tissue layers, this study developed an optical tissue clearing (OTC) laser probe (OTCLP) system which can utilize four different OTC methods: 1) tissue temperature control from 40 to 10°C; 2) laser pulse frequency from 5 to 30 Hz; 3) glycerol injection at a local region; and 4) a combination of the aforementioned three methods. The efficacy of the OTC methods was evaluated and compared by investigating laser beam profiles in *ex-vivo* porcine skin samples. Results demonstrated that total (peak) intensity at full width at half maximum of laser beam profile when compared to control data was increased: 1) 1.21(1.39)-fold at 10°C; 2) 1.22 (1.49)-fold at a laser pulse frequency of 5 Hz; 3) 1.64 (2.41)-fold with 95% glycerol injection; 4) 1.86 (3.4)-fold with the combination method. In conclusion, the OTCLP system successfully improved the laser photon density in deep tissue layers and may be utilized as a useful tool in LLLT by increasing laser photon density.

Keywords : Photon density, Low-level laser therapy, Laser modulation, Optical tissue clearing

OCIS codes : (000.2170) Equipment and techniques; (120.4820) Optical systems; (170.1610) Clinical applications; (350.4600) Optical engineering

I. INTRODUCTION

In low-level laser therapy (LLLT), optical tissue clearing (OTC) has been considered as a useful method to enhance therapeutic efficacy by minimizing tissue turbidity, which limits the depth of light penetration and photon density in soft tissue [1, 2]. Although various OTC methods, such as mechanical (positive pressure and stretching), chemical (hyperosmotic chemical agent [HCA]), and thermal (temperature) methods, have been studied to minimize tissue turbidity by reducing light scattering in soft tissue [3-6], the clinical applications have not been sufficiently studied to date.

Thermal methods are based on the mechanism of gel/liquid-crystalline phase transitions of all lipids in human subdermis [7, 8], modifications of collagen fiber structure in human dermis [9], degradation of cellular components [10], and

variation of the refractive index of intercellular fluid [11]. Khalil *et al.* [12] demonstrated that tissue-cooling can increase light penetration depth in human skin due to the effects of temperature on the absorption and reduced scattering coefficients. Such previous studies support that light scattering in the human dermis layer can be decreased by decreasing tissue temperature and, therefore, an OTC effect may be achieved, resulting in increasing the photon density in deep tissue layers.

Since Tuchin *et al.* [13] first reported an OTC method using chemical agents, numerous investigations have been performed to understand the OTC mechanism, such as skin dehydration, partial replacement of the interstitial fluid by the immersion substance, structural modification or dissociation of collagen, and a combination of the two [13-17]. Rylander *et al.* [18] and Yu *et al.* [19] suggested

*Corresponding author: bjung@yonsei.ac.kr

that skin dehydration is the prominent mechanism of OTC and Yeh *et al.* [20] suggested that the reversible change of both collagen structure and size can affect OTC. In recent *in-vivo* studies, Ghosn *et al.* monitored and quantified the permeability rate of glucose solutions in *in-vivo* monkey skin by using OCT [21], and Wen *et al.* reported that the thickness decrease of dermis and regular arrangement of tissue fibers were prominent OTC mechanisms [22]. Tuchin clearly described the clinical diagnostic efficacy of chemical agents [23, 24]. Recently, both Yoon *et al.* and Kang *et al.* reported that the combination of glycerol, compression, micro-needling, and sonophoresis decreased the diffusion time of glycerol and had a greater OTC effect than independent application [3-6]. Liu *et al.* investigated various light irradiations to increase permeability of chemical agents and, therefore, enhance the efficacy of OTC [25].

Although many studies have been reported to evaluate the effect of laser pulse frequency (LPF) on LLLT [26], the efficacy of LPF [continuous wave (CW) or pulsed wave (PW)] on LLLT is still unclear, and there is no complete consensus for the mechanism of LLLT. However, it was generally confirmed that the PW laser may penetrate deeper into soft tissue as compared to the CW [26, 27]. The PW from 2.5 to 10,000 Hz on LLLT was frequently used in the clinic due to the deep light penetration depth in soft tissue [26].

In this study, an optical tissue clearing laser probe (OTCLP) system was developed to utilize simultaneously or independently the three OTC methods of tissue temperature, LPF, and injection of glycerol. The OTCLP system was characterized by quantitatively evaluating OTC effects in *ex-vivo* porcine skin samples (PSSs).

II. METHODS

2.1. Sample Preparation

Ex-vivo abdominal PSSs were used due to the similar structural and immunohistochemical characteristics as human skin [28-30]. The PSSs were obtained from a local slaughter house and prepared to a size of $20 \times 20 \text{ mm}^2$ with an average thickness of 1.89 mm without a fat layer. Although the time period of each experiment was not recorded, it was possibly shortened in order to minimize the dehydration of PSSs during experiments. Five different PSSs were used at each experiment of Section 2.4 to ensure the reproducibility of experiment results. Room temperature was maintained at 19°C and the temperature of PSSs was monitored with a digital thermometer.

The studies of section 2.4.1 through 2.4.3 were separately investigated with different PSSs to obtain optimal OTC parameters. The study of section 2.4.4 has utilized the optimal OTC parameters which were sequentially applied to an identical PSS in order to minimize the variation of tissue conditions as a function of time.

2.2. Tissue Optical Clearing Laser Probe (OTCLP)

The OTCLP system was developed to increase the laser photon density (LPD) in soft tissue. Fig. 1(a) shows a schematic diagram of the OTCLP which consists of an air suction hole to supply negative compression for maintaining attachment, transparent acrylic body in order to observe skin deformation, and a metal supporter for both guiding an optical fiber and internal water circulation. The metal supporter was made from aluminum for high thermal conductivity. Fig. 1(b) shows the picture of the self-sustaining OTCLP, which can independently or simultaneously control tissue temperature,

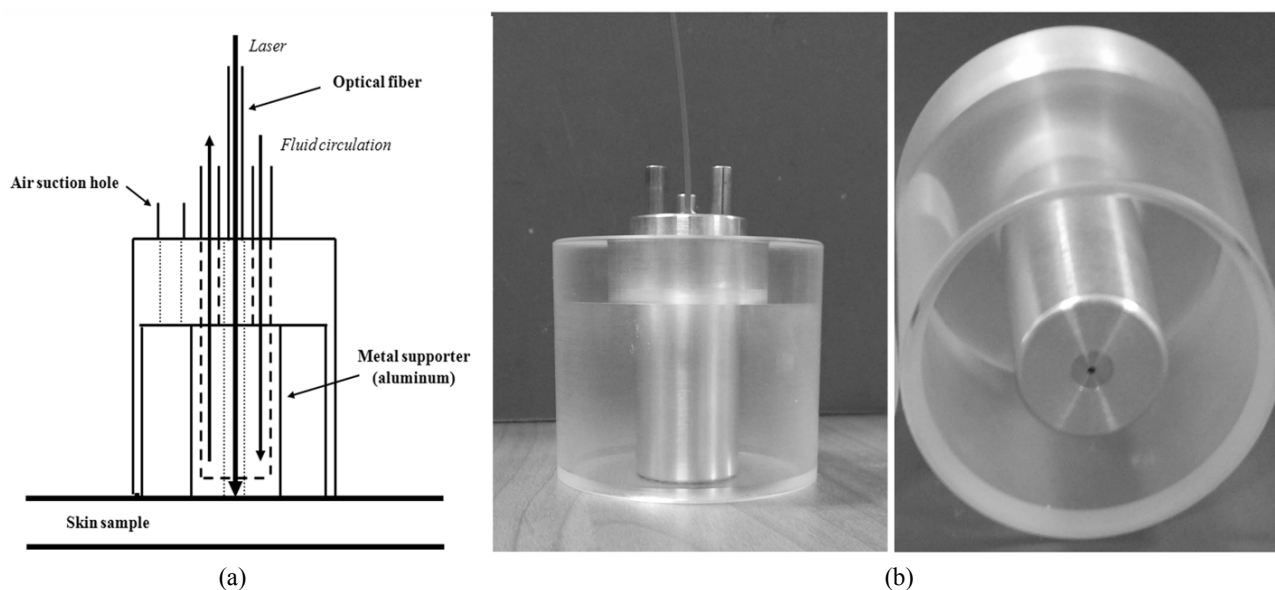


FIG. 1. (a) Schematic diagram and (b) photograph of tissue optical clearing laser probe which consists of an air suction hole to supply negative compression and a metal supporter for both guiding an optical fiber and circulating temperature-controlled water.

LPF, and negative compression.

2.3. Experimental Setup

Figure 2 shows an experimental setup to obtain the diffusion images of the laser beam profile (LBP) which are transmitted PSSs. It consists of the compartments of diode laser, image acquisition, and temperature control.

The temperature control compartment has a refrigerating bath circulator (RW-0540G; Jeio-tech, Daejeon, Korea) to provide temperature-controlled water. The diode laser compartment consists of a 660 nm AlGaInP diode laser (HL6545MG; Thorlabs, Kansas, USA), diode laser mount (LDM21; Thorlabs, Kansas, USA) equipped with aspheric lenses (C280TME-B; A240TM-B; Thorlabs, Kansas, USA), TEC controller (TCM1000T; Thorlabs, Kansas, USA), laser diode driver (IP500; Thorlabs, Kansas, USA), and optical chopper (SR540; Stanford Research Systems, California, USA). This compartment controls the irradiation mode (PW or CW) of the low-level laser which is delivered to PSSs by a multi-mode optical fiber (FT400EMT; Thorlabs, Kansas, USA). Maximum laser power was adjusted to 10 mW at the distal end of the optical fiber in order to avoid light saturation in the LBP image. The image acquisition compartment consists of a CCD camera (XC-HR57; Sony, Tokyo, Japan) to obtain the LBP images of transmitted PSSs.

A double-integrating sphere system (Avasphere-30; Avantes, Apeldoorn, Netherlands) [31-33] which is equipped with a Quartz Tungsten-Halogen lamp source and a fiber optics spectrometer (USB4000; Ocean Optics, Florida, USA) was used to measure optical properties of PSSs as a function of tissue temperature. The data at 660 nm from the measured spectrum were used because this study used a 660 nm diode laser.

2.4. Experiment Procedure

A combination and three independent methods were

applied to the PSSs to obtain LBP images: 1) as a function of tissue temperature ranging from 40 to 10°C at 10°C decrements; 2) as a function of the LPFs ranging from 5 to 30 Hz at 5 Hz increments; and 3) immediately after injection of 95% glycerol in the region of interest (ROI); and 4) by applying a combination method of the aforementioned three methods. The LBP image of 251×251 pixels was extracted and analyzed with a laboratory-built MATLAB program. The OTC effects of each method were quantitatively evaluated by computing the peak intensity (PI) and the total intensity (TI) which is defined as the intensity integration at full width at half maximum (FWHM) on LBP.

2.4.1. Tissue Temperature

LBPs were obtained as a function of tissue temperatures from 40 to 10°C at 10°C decrements. The LBP obtained at 40°C was used as control data and the others as test data. The temperature of PSSs was controlled by circulating temperature-controlled water through the metal supporter of the OTCLP.

In order to investigate optical property variation due to tissue temperature, the PSSs were maintained at the temperature of interest in a refrigerating bath circulator before measurement, and then measurements were performed immediately after the PSSs were exposed to room temperature. Both transmittance and reflectance of PSSs were measured as a function of tissue temperature. Reduced scattering coefficients (μ'_s) [35] were computed with an Inverse-Adding Doubling program [35, 36].

2.4.2. Laser Pulse Frequency (LPF)

LBP images of PSSs were obtained at 50 frames/sec as a function of LPF. CW and PW irradiation modes were used as control and test data, respectively. The LBP images with maximum PI were selected at each LPF and used for quantitative analysis of LBP.

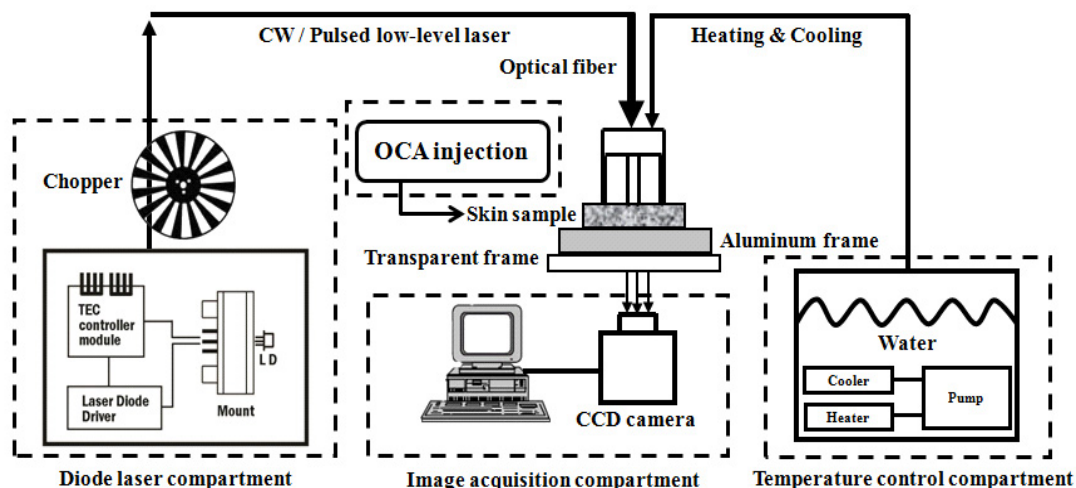


FIG. 2. Experimental setup to obtain laser beam profile in ex-vivo porcine skin sample. It consists of compartments of diode laser, image acquisition, and temperature control.

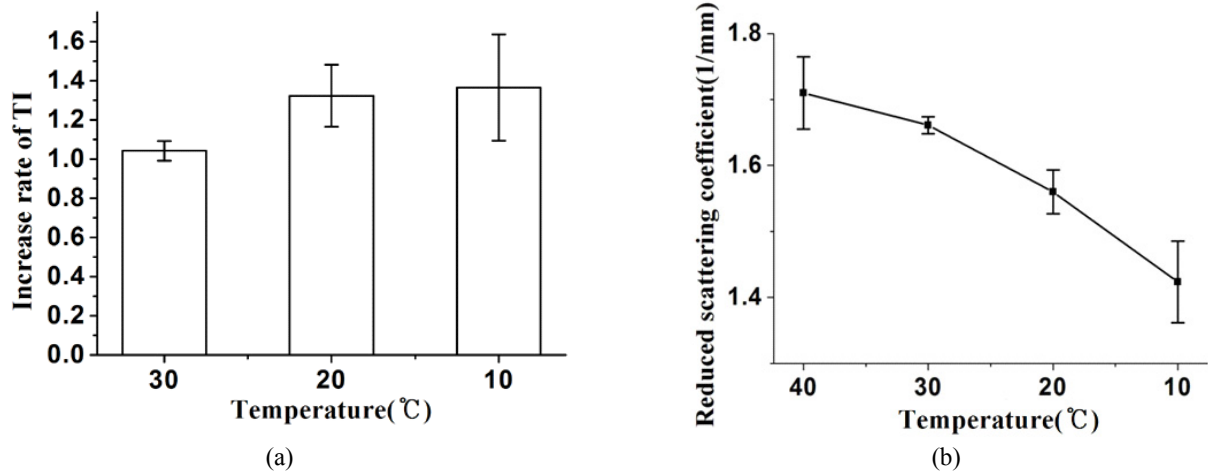


FIG. 3. (a) Total intensity (TI) at full width at half maximum and (b) reduced scattering coefficient as a function of tissue temperature in ex-vivo porcine skin samples. In the graph, the error bar indicates standard deviation.

2.4.3. 95% Glycerol Injection

95% glycerol was injected at a ROI where the laser was irradiated and LBP images were obtained immediately after glycerol injection. The PSSs with and without glycerol injection were used as control and test data, respectively.

2.4.4. Comparison of Independent and Combination Methods

The optimal OTC parameters for the combination method were derived from the best results of the three independent methods (section 2.4.1 through 2.4.3). The LBP for control data was first obtained at the tissue temperature of 40°C, and then independent and combination methods were sequentially applied to obtain the LBPs for test data as follows: 1) the optimal LPF laser was applied at the tissue temperature of 40°C; 2) 95% glycerol was injected and then the CW laser was applied at the tissue temperature of 40°C; 3) tissue temperature was decreased to optimal tissue temperature and then the CW laser was applied at the region without glycerol injection; and 4) the combination method was finally applied.

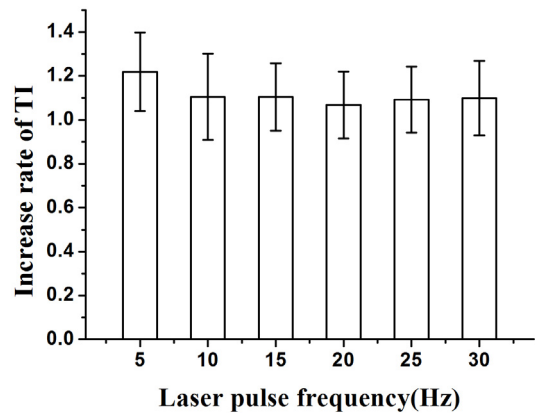


FIG. 4. Total intensity (TI) at full width at half maximum for laser pulse frequencies ranging 5 to 30 Hz in ex-vivo porcine skin samples. In the graph, the error bar indicates standard deviation.

III. RESULTS

3.1. Tissue Temperature

The laser intensity was increased by decreasing the temperature of PSSs, and the maximum and minimum laser intensities were observed at 10°C and 40°C, respectively. Fig. 3(a) shows that average TI were 1.04-, 1.32-, and 1.37-fold higher at 30, 20, and 10°C when compared to 40°C, respectively. Fig. 3(b) shows the μ'_s as a function of temperature that resulted in 1.71 mm⁻¹ at 40°C, 1.66 mm⁻¹ at 30°C, 1.56 mm⁻¹ at 20°C and 1.42 mm⁻¹ at 10°C.

3.2. Laser Pulse Frequency

Figure 4 shows an average increase rate of TI at each LPF in which 5 Hz was determined to have the highest

increase rate of 1.22-fold. Other LPFs presented a negligible increase rate of 1.11-fold at 10 Hz, 1.10-fold at 15 Hz, 1.07-fold at 20 Hz, 1.09-fold at 25 Hz, and 1.10-fold at 30 Hz.

3.3. 95% Glycerol Injection

Figure 5 shows the increase rate of PI and TI in 95% glycerol injected PSSs that were used as test data while a non-injected PSS was used as control. The LBP of test data resulted in a remarkable enhancement of laser transmission. It showed that PI and TI were 2.13-fold and 1.81-fold higher in test than control data, respectively.

3.4. Comparison of Independent and Combination Methods

Figure 6 shows an increase rate of PI and TI for: (1) LPF of 5 Hz, (2) 95% glycerol injection, (3) tissue temperature of 10°C, and (4) a combination of the aforementioned three methods. Fig. 6(a) shows that the PI of the combination

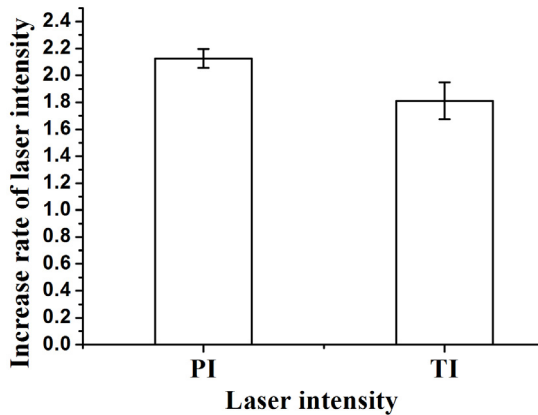


FIG. 5. The peak intensity (PI) and total intensity (TI) at full width at half maximum for 95% glycerol injection. In the graph, the error bar indicates standard deviation.

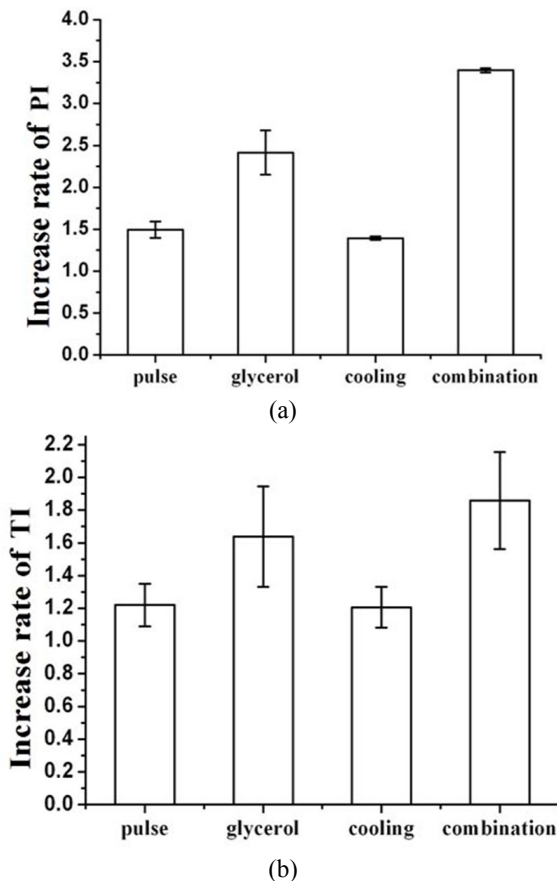


FIG. 6. Comparison of (a) peak intensity (PI) and (b) total intensity (TI) at full width at half maximum for tissue optical clearing methods with laser pulse frequency of 5 Hz, 95% glycerol injection, tissue temperature of 10°C, and their combination. In the graph, the error bar indicates standard deviation.

method (3.40-fold) was higher than LPF (1.49-fold), glycerol injection (2.41-fold), and tissue cooling (1.39-fold) methods. Fig. 6(b) presents that the TI of the combination method

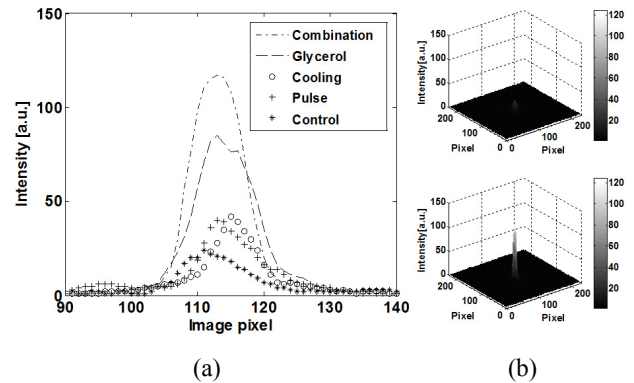


FIG. 7. Comparison of (a) 2D and (b) 3D laser beam profiles for control (upper) and the combination method (lower) of tissue cooling of 10°C, laser pulse frequency of 5 Hz, and 95% glycerol injection.

(1.86-fold) was also higher than LPF (1.22-fold), glycerol injection (1.64-fold), and tissue cooling (1.21-fold).

3.5. Comparison of Laser Beam Profiles

Figure 7(a) shows 2D LBPs of tissue cooling (10°C), LPF (5 Hz), 95% glycerol injection, the combination method, and the control. The 2D LBPs showed that OTC methods resulted in higher PI than the control, and the combination method resulted in relatively higher PI than the independent methods. Fig. 7(b) illustrates 3D LBPs of the control (upper) and the combination method (lower) and graphically presents a much greater OTC effect of the combination method.

IV. DISCUSSION

LLLT has been primarily used in wound healing, tissue regeneration, pain and inflammation relief, prevention of tissue death, and mitigation of neurological degeneration [3]. However, tissue turbidity decreases the therapeutic efficacy of LLLT due to light scattering in soft tissue. In order to enhance tissue clarity, various OTC methods have been investigated [1, 2]. Compression, tissue cooling, and HCA application were suggested as effective OTC methods to minimize light scattering in soft tissue. In this study, the OTCLP system was developed for independent or simultaneous application of OTC methods of tissue temperature, LPF, and glycerol injection in the ROI.

The OTC results from independent methods in sections 3.1 through 3.3 were slightly different from those in section 3.4 although an identical OTCLP system was used. For example, OTC effects at 10°C were increased 1.37-fold in section 3.1 and 1.21-fold in section 3.4. For comparison of OTC effects (Figs. 6 and 7), the independent and combination methods used different PSSs as described in section 2.1. Therefore, such differences might be caused by different thicknesses and dehydration of PSSs and

different residue of the fat layer which might affect the optical properties of PSSs.

Although the optical property of PSS, μ_a , was not presented here because its variation was negligible from 40°C (0.0325 mm⁻¹) to 10°C (0.0431 mm⁻¹), it showed an increasing pattern with decreasing temperature. Fig. 3 demonstrates that decreasing the tissue temperature resulted in decreasing the μ 's and, therefore, increasing the PI and TI which means enhancement of photon density in the dermis. As described in previous studies, such results may be caused by the modification of collagen fiber structure, degradation of cellular components, and change of the refractive index of intercellular fluid [15-20].

A previous study described that a PW laser may have a deeper penetration depth compared to the CW laser [26]. In this study, the TIs of the PW laser increased more than about 10% compared to that of the CW laser (Fig. 4), but the increase rates of TI may be negligible because they are closely within the error bars. As a result, the penetration depth of the laser may not be strongly affected by the LPFs except for at 5 Hz which presented about 20% of the increase rate of TI in this study (Fig. 4). The clinical efficacy of LLLT may not depend on only the penetration depth of laser [26]. Therefore, the use of the CW laser or PW laser should be carefully determined depending on the therapeutic purposes.

Figure 5 shows the rapid OTC effect of locally injected glycerol. The LBP remarkably increased when compared to control. Skin dehydration and collagen dissociation may cause the reduction of light scattering by minimizing refractive index mismatching [15-17]. Recently, Yu *et al.* [19] reported that the dehydration induced by an optical clearing agent is a prominent mechanism for improving tissue optical transmittance. Direct injection of glycerol may have a clinical advantage of an immediate OTC effect in local treatment. Deng *et al.* [37] recently reported that the OTC efficacy by glycerol has negligible variation in the temperature range from 4 to 25°C. Therefore, the temperature in OTC efficacy was not considered in this study because glycerol solution and PSSs were kept at 19 and 10°C, respectively.

The combination method presented the greatest OTC efficacy compared to other methods (Fig. 6), and it was graphically confirmed with 3D LBPs (Fig. 7). The combination method was successfully integrated in the OTCLP system. Mechanical tissue compression, which is one of the effective OTC methods [6, 38-41], was not integrated into the OTCLP system because of the structural difficulty of the OTCLP system. The combination method presented similar increase rate of PI when compared to mechanical compression [6] applied using a 40 mm probe diameter with a negative compression of 30kPa. It is expected that if the mechanical compression method [6] is integrated into the OTCLP system, the laser photon density may be further enhanced in deeper tissue.

Comparisons of OTC methods may be valuable but are not easy because of differences in experimental setups and

analysis methods [3, 5, 6, 9, 11, 12, 32, 33, 37-40]. However, Izquierdo-Román *et al.* reported that mechanical OTC methods are more effective about 2-3 fold than the chemical OTC method and achieve up to a 4-fold OTC increase [40], which is comparable to the 3.4-fold OTC increase of the combination OTC method.

V. CONCLUSION

In this study, the OTC efficacy was quantitatively analyzed and compared by manipulating OTC methods, such as tissue temperature, glycerol injection, and LPF in soft tissue. Independent OTC methods were integrated into an OTCLP system in order to enhance and optimize OTC efficacy by controlling OTC factors. In future study, if a mechanical compression method is added on the OTCLP system, the efficacy of OTC may be further enhanced. It is expected that the OTCLP system may be clinically used as a non-invasive tool in low-level laser therapy (for instant, pain, wound healing, and laser acupuncture) of deeper tissue due to the increase of photon density.

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