In vitro study of ultrasound and different-concentration glycerol-induced changes in human skin optical attenuation assessed with optical coherence tomography

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Abstract. Previous studies have demonstrated the ultrasound-induced skin optical clearing enhancement with topical application of 60% glycerol (G) on *in vitro* porcine skin and *in vivo* human skin. Our purpose was to find the relation between the effect of optical skin clearing and different concentrations of glycerol and to find more effective ultrasound-glycerol combinations on optical skin clearing. The enhancement effect of ultrasound [Sonophoresis (SP) delivery] in combination with 40% G, 60% G, and 80% G on *in vitro* human skin optical clearing was investigated. Light imaging depths of skin were measured using optical coherence tomography. Different concentrations of glycerol and ultrasound with a frequency of 1 MHz and an intensity of 0.5 W/cm² was simultaneously applied for 15 min. The results show that with the increase of concentration of glycerol, the optical clearing of skin is much improved. Optical clearing capability of glycerol was more enhanced with simultaneous application of ultrasound compared with glycerol alone. The attenuation coefficients of skin tissues after application of 40% G/SP, 60% G/SP, and 80% G/SP decreased approximately 11.8%, 18.5%, and 20.0% at 15 min compared with 40% G, 60% G, and 80% G alone, respectively. The greatest decrease in attenuation coefficients at 60 min was approximately 52.3% and 63.4% for 80% G (without ultrasound) and 80% G/SP (with ultrasound), respectively, which are 2.1-fold and 2.6-fold to that in the 40% G. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3432750]

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1 Introduction

Engineered tissue optics is a new research area that allows reversibly altering light scattering and absorption within naturally turbid tissues in a precise and controlled manner.¹ *Tissue optical clearing* permits delivery of near-collimated light deeper into tissue, potentially improving the capabilities of various optical diagnostic and therapeutic techniques such as new light-based therapies for reshaping or removing adipose tissue.¹ The effects of optical clearing of highly scattering biological tissue by using the optical clearing agents (OCAs)—e.g., glycerol (G),^{2–4} glucose,^{5–7} and dimethyl sulfoxide^{8,9}—have been investigated using many techniques, examples of which include near-infrared (NIR) spectrophotometers^{10,11} and optical coherence tomography (OCT) imaging.^{12–17} OCT is a recently developed imaging technique that has the potential for early diagnosis of diseases in human tissues.^{18,19} It has very high axial resolution, but the

high scattering nature of nontransparent human tissue limits the imaging depth of OCT to 1 to 2 millimeters. Although the depth of light penetrations of biotissue can be effectively increased by OCAs, however, the outermost layer of the skin, the stratum corneum (SC), presents a significant barrier to the most topically applied OCAs and is hence responsible for the poor optical clearing effect.^{17,20,21} To reduce barrier function of skin tissues during optical clearing, many different chemical and physical methods have been proposed. Chemical penetration enhancers,²² photothermal,^{23,24} sandpaper,²⁵ microneedle,^{26,27} electroporation,²⁸ iontophoresis,²⁹ tape stripping,³⁰ laser ablation,³¹ ultrasound [sonophoretic (SP) delivery],^{32–36} and jet injection have been shown to accelerate the skin permeability of OCAs.²² Recent investigation indicated that sonophoretic delivery, as a noninvasive physical method, exhibited an enhancing skin clearing effect when applied topically with OCAs.^{16–18} Significant ultrasound-induced enhancement in OCT imaging depth and contrast of *in vitro* porcine skin and *in vivo* human skin was found,^{16,18} but it was just research on 60% glycerol in combination with

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Table 1 Treatment in this study.

Treatment	Glycerol	Distilled water	Ultrasound (SP)	Refractive index (n)	Cases
40% G	40	60	–	1.3941	6
40% G/SP	40	60	+	1.3941	6
60% G	60	40	–	1.4190	6
60% G/SP	60	40	+	1.4190	6
80% G	80	20	–	1.4494	6
80% G/SP	80	20	+	1.4494	6

ultrasound. In this study, we use the attenuation coefficients within the skin tissues to evaluate the optical skin clearing change after topical application of different concentrations of glycerol and compare with different concentrations of glycerol in combination with 15-min ultrasound at various time intervals of 0, 15, 30, 45, and 60 min, respectively. The aim of this investigation is to find the relation between effect of optical skin clearing and different concentrations of glycerol and explore a more effective method for skin optical clearing than pure OCAs.

2 Materials and Methods

2.1 Tissue Preparation

A total of 36 normal human skin tissue samples were taken from the abdominal skin tissue of 36 human subjects immediately after surgical excision of subcutaneous fatty tissue. Each tissue sample was immediately rinsed briefly in saline to remove excess surface blood after the excision. Then, the tissue sample was placed in a bottle with saline as soon as possible, sealed to prevent natural dehydration, and stored in a refrigerator at -70°C . Each skin sample was cut into $27.5\text{ mm} \times 27.5\text{ mm}$ samples by microtome before measurement (the mean thickness of samples was $1.83 \pm 0.05\text{ mm}$) and then thawed to room temperature (25°C).

2.2 OCT System

The OCT system used in this study consists mainly of re-source module, fiber conduction module, reference arms module, and sample arms module. A schematic of the OCT system was shown in the literature.³⁷ The broadband light source is a superluminescent diode with a central wavelength at 1310 nm and a bandwidth of 50 nm. The light source yields a $15\text{-}\mu\text{m}$ axial resolution in free space, or approximately $10\text{ }\mu\text{m}$ in tissue, as the mean refractive index of bulk tissue is assumed to be 1.38. The transverse resolution of the system is about $25\text{ }\mu\text{m}$, as determined by the focal spot size produced by the probe beam. The signal-to-noise ratio (SNR) of this system is measured at 100 dB. A visible light source ($\lambda=645\text{ nm}$) was used to guide the probe beam. The OCT system operation is controlled automatically by computer. The detector current is demodulated using a lock-in amplifier and a low-pass filter in software prior to storage. Each in-depth scanning (A-scan) consists of 10,000 data points. The lateral scanning (B-scan)

image is obtained by moving the mirror relative to the tissue sample, which takes about 1.0 s. The data acquisition software is written in LabView 7.2-D. OCT images are obtained in each experiment and stored on the PC for further processing.^{37,38}

2.3 Ultrasound and Glycerol Application

A BM628 sonicator with a 0.8-cm-diam probe (Guangzhou Radium Cosmetic Co. Ltd., Guangzhou, China) was used. The ultrasound frequency and intensity were 1 to 1.1 MHz and 0.5 W/cm^2 , respectively. Sonicators with this frequency are most commonly used in cosmetic treatments and transdermal drug delivery (TDD).

Glycerol (with 99.9% concentration) was purchased from the Tianjin Damao Chemical Reagent Factory (China). The concentration of the glycerol solutions used in this study was reduced to 40%, 60%, and 80% through mixing the agents with distilled water (vol/vol). For the glycerol–ultrasound groups, solutions of 40%, 60%, and 80% glycerol were applied to the surface of each skin sample, respectively, and ultrasound was simultaneously performed on these samples for a period of 15 min, respectively. During ultrasound treatment, the ultrasound probe was immersed in the glycerol solution that was topically applied to the surface of skin. After the 15-min ultrasound treatment, the sample was kept occluded with solution for a total of 60 min. The optical clearing agents and ultrasound treatments are listed in Table 1.

2.4 Normalized Intensity Profiles and Attenuation Coefficient

The OCT normalized intensity profiles were obtained by averaging the linearized signal intensity across the lateral imaging range as a function of depth. A best-fit exponential curve covering the epidermis and dermis in depth was applied to the averaged data of each group.

OCT imaging is based on the difference of backscattered light. The result is the measurement of optical backscattering or reflectance, $R(z)$, versus axial ranging distance, or depth, z . The reflectance depends on the optical properties of tissue—i.e., the absorption (μ_a) and scattering (μ_s) coefficients, or total attenuation coefficient (μ_t), $\mu_t = \mu_a + \mu_s$ (Ref. 39). The relationship between $R(z)$ and μ_t is, however, very complicated due to the high-scattering nature of biological tissue. However, for relatively transparent tissue, the reflected power

Fig. 1 OCT images of skin tissue at 0, 15, 30, 45, and 60 min (from right to left) after topical application of (a) 40% G, (b) 60% G, and (c) 80% G alone, respectively.

will be negatively proportional to $2\mu_t z$ in exponential scale according to the single scattering model, which is valid for an optical depth less than 4 (Refs. 39 and 40):

$$R(z) = I_0 \times T(z) \times \exp(-2\mu_t z), \quad (1)$$

where I_0 is the light intensity launched into the tissue sample, and $T(z)$ is the reflectivity of the tissue at depth z . The factor of 2 in the exponential accounts for the light passing through the tissue twice after being backscattered. It should be noted that the optical depth is a measure of the depth in terms of the number of mean free path lengths—i.e., $\mu_s z$ (Ref. 39). $T(z)$ is linked to the local refractive index and backscattering property of the tissue.^{41–43} However, for a homogenous turbid medium, it can be considered to be constant. Therefore, μ_t can be obtained theoretically from the reflectance measurements at two different depths, z_1 and z_2 (Ref. 39):

$$\mu_t = \frac{1}{2(\Delta z)} \ln \left[\frac{R(z_1)}{R(z_2)} \right], \quad (2)$$

where $\Delta z = |z_1 - z_2|$. As noise is inevitable in the measurement, a final result should thus be obtained by the use of a least-squares fitting technique in order to improve the accuracy of the determined value of μ_t . The total attenuation coefficients of light through skin tissues are calculated by Eq. (2).

Reduction of attenuation coefficients $\Delta\mu_t$ by the solutions at the time intervals of treatment were calculated according to

$$\Delta\mu_t = \frac{\mu_{t(\text{treated})} - \mu_{t(\text{control})}}{\mu_{t(\text{control})}} \times 100\%, \quad (3)$$

where μ_t is the attenuation coefficient. The subscripts *control* and *treated* refer to the samples before (0 min) and after the application of agents at the different time intervals, respectively.

2.5 Statistical Analysis

The data from all samples were presented as means $\pm$ SD and analyzed by an SPSS 10.0 software paired-test. The $p < 0.05$ value indicated significant difference.

3 Results and Discussion

3.1 Without Ultrasound

Figures 1(a)–1(c) are the OCT images of *in vitro* human skin at 0, 15, 30, 45, and 60 min after topical application of 40% G, 60% G, and 80% G alone, respectively. It can be seen that light penetration is limited in native skin (see Fig. 1; 0 min). It is shown that structural features within the skin are more visualized with the increase of time after different treatments, and the light penetration was enhanced, as the light can be seen coming back from the deeper skin with the increase of time after treatment. The high-concentration solutions induce more transparency of skin than the surroundings, so the clearing capability of 80% G was most improved over that of 40% G and 60% G alone, whereas 40% G has the lowest clearing effect (Fig. 1). This means that with the increase of concentration of glycerol, the optical clearing of skin is more improved. The results obtained were similar to these of Mao et al., who have demonstrated more optical skin clearing improvement than that with low-concentration glycerol injected into the dorsal dermas of SD rat from reflectance spectra.⁴⁴

3.2 With Ultrasound

The dynamics of enhancement in OCT normalized intensity profiles with corresponding exponential best-fit curves of skin at 0, 15, and 30 min after topical application of 40% G/SP, 60% G/SP, and 80% G/SP are shown in Fig. 2. It can be seen from Fig. 2 that due to enhanced light transport within the skin tissue, more light propagates to the reflective surface underneath the skin tissue, giving rise to a stronger back-reflected signal in the OCT amplitude data. The degree of change increases with the increase of concentration of glycerol, meaning that the largest increases in the OCT normalized intensity profiles of skin after treatment occur with 80% G/SP rather than that of 40% G/SP and 60% G/SP over the same time [Figs. 2(a)–2(c)].

3.3 Without and with Ultrasound

Figure 3 summarizes the attenuation coefficients of skin tissue at 0, 15, 30, 45, and 60 min after topical application of (a) 40% G, 60% G, and 80% G (without ultrasound) and (b) 40% G/SP, 60% G/SP, and 80% G/SP (with ultrasound), respec-

Fig. 2 OCT normalized intensity profiles with corresponding exponential best-fit curves of *in vitro* human skin at 0, 15, and 30 min after topical application. (a) 40% G/SP, (b) 60% G/SP, and (c) 80% G/SP.

tively. It can be seen from Fig. 3 that not only without ultrasound, but also with ultrasound, the light attenuation coefficients of skin tissue decrease with the increase of time after topical application—e.g., for 40% G/SP, the light attenuation coefficients of skin tissue are 1.97, 1.49, 1.32, 1.29, and 1.26 mm⁻¹ at 0, 15, 30, 45, and 60 min, respectively. It also can be seen that the attenuation coefficients of skin tissue at the same times decreases with the increase of concentration of glycerol. For example, the attenuation coefficients of skin tis-

sue at 15 min after topical application of 40% G, 60% G, and 80% G alone are (1.69 ± 0.05) , (1.58 ± 0.04) , and (1.41 ± 0.04) mm⁻¹, respectively; the light attenuation coefficients of skin tissue at 15 min after treatment with of 40% G/SP, 60% G/SP, and 80% G/SP are (1.49 ± 0.04) , (1.28 ± 0.03) and (1.14 ± 0.03) mm⁻¹, respectively. These results agree with previous research that the glycerol solutions with high concentration induced much decreased reflectance,

Fig. 3 Attenuation coefficients of skin tissues at 0, 15, 30, 45, and 60 min after topical application of (a) 40% G, 60% G, 80% G alone (without ultrasound) and (b) 40% G/SP, 60% G/SP, 80% G/SP (with ultrasound).

Fig. 4 Attenuation reduction in human skins induced by different concentrations of glycerol and (a) without ultrasound and (b) with ultrasound.

which means that the μ_s of tissue decayed a lot and the skin became much optical clearing.^{45,46} Comparing glycerol-ultrasound group with the same concentration of glycerol alone group, the attenuation coefficients of skin tissue after application of 40% G/SP, and 60% G/SP, and 80% G/SP decreased approximately 11.8%, 18.5%, and 20.0% at 15 min over that of 40 %G, 60 %G, and 80 %G alone, respectively. This means that there is a large change in the attenuation coefficients with ultrasound and without ultrasound. It also clearly demonstrates the enhancement of optical skin clearing when ultrasound is applied. This phenomenon is consistent with previous findings that ultrasound caused the increase of cell membrane permeability in TDD.^{47–50}

The quantitative reductions of attenuation coefficients at 60 min according to Eq. (3) for skin samples under different treatment procedures (40% G, 60% G, 80% G, 40% G/SP, 60% G/SP, and 80% G/SP) are shown in Figs. 4(a) and 4(b), respectively. The greatest decreases in attenuation coefficients were approximately 52.3% and 63.4% for 80% G (without ultrasound) and 80% G/SP (with ultrasound), respectively, which are 2.1-fold and 2.6-fold that in the 40% G at 60 min. The differences in enhancement in transmission of skin tissue are significant between 60% G and 40% G at 30 min ($P < 0.05$); between 80% G and 60% G at 30 min ($P < 0.05$); between 60% G/SP and 40% G/SP at 30 min ($P < 0.05$); between 80% G/SP and 60% G/SP at 30 min ($P < 0.05$); between 40% G/SP and 40% G at 15 min ($P < 0.05$); between 60% G/SP and 60% G at 15 min ($P < 0.05$); and between 80% G/SP and 80% G at 15 min ($P < 0.05$). Figures 3 and 4 clearly demonstrate that there is a more obvious enhancement effect for skin optical clearing when ultrasound is applied than that without ultrasound. These results are consistent with Xu's findings of *in vitro* porcine skin clearing enhancement of 60% glycerol in combination with ultrasound.^{16–18,51}

Ultrasound has demonstrated an enhancing transdermal mass transport effect when applied topically in TDD. Its mechanism is believed to be through acoustic cavitation, which is the formation and collapse of gaseous cavities, and has the dominant role in sonophoresis.^{52,53} Based on the aforementioned results, it indicated that due to ultrasound's penetration enhancing and convection effect, more glycerol can penetrate the skin tissues and glycerol can penetrate more quickly to achieve more refractive index matching in human

skin tissue *in vitro* to improve skin tissue optical clearing.

It is necessary to note that the diffusion of glycerol in human skin tissue will change the refractive index during the course of the experiment and therefore will affect the size of the region selected for calculations. But this alteration in tissue optical size was small enough and was neglected in our computation. Also, biological tissue is a turbid nonuniform tissue with some variations in the refractive indexes through its layers.⁵⁴

4 Conclusion

We have summarized the optical clearing of human skin after application of different-concentration glycerol and analyzed the difference effect in optical skin clearing after treatment with ultrasound and without ultrasound. The results reported here indicate that glycerol with high concentration can markedly enhance optical clearing of skin. But it was confirmed that simultaneous application of ultrasound and glycerol led to an obvious increase in light transmission and imaging depth through *in vitro* human skin. Our future studies will be focused on increasing knowledge of the mechanisms for synergistic effect of ultrasound and glycerol by microscopy or other advanced techniques. There is a stronger correlation with insonation frequency and power density, and there is a difference between *in vivo* and *in vitro* skin; therefore, in order to more safely and effectively improve optical skin tissue clearing, future studies should also focus on these correlations.

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