Improvement of skin optical clearing efficacy by topical treatment of glycerol at different temperatures

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Abstract: In the past decades, laser has been widely used in clinical diagnosis and cosmetic therapy. However, there is limitation for further usage in deeper tissue for high scattering property. Skin optical clearing technique, by introducing optical clearing agents (OCAs) into tissue, will have a potential impact on optical diagnosis and therapy. In this work, anhydrous glycerol at different temperatures of 4, 25, 32 and 45ºC were applied respectively to in vitro porcine skin, and reflectance and transmittance spectra were then measured dynamically using a spectrometry combined with integrating sphere system. Further, reduced scattering coefficient and penetration depth were obtained. Results showed that, glycerol at different temperatures could induce the reduced scattering coefficient of in vitro skin to decrease and the penetration depth to increase. 4 and 25ºC glycerol had similar effect, decreasing the scattering by 48.2% and 49.7%, and increasing penetration depth by 37.9% and 39.5%, respectively. However, 32 and 45ºC glycerol treatment could decrease scattering by 61.6% and 76.6%, and increase penetration depth by 53.3% and 84.1%, respectively. In conclusion, glycerol at higher temperature can induce greater and faster skin optical clearing efficacy.

1. Introduction
High scattering property of biological tissue for VIS-NIR light limits the penetration depth of light deep into tissue, which has become the obstacle of optical diagnosis and therapy [1]. However, the tissue scattering can be reduced and the penetration depth can be improved by injecting agents with characteristics of high permeability, high refractive index and biocompatibility into tissues, which is called tissue optical clearing technique and was proposed by Tuchin VV [2-4].

Skin is located on the out surface of organisms and has an important role for clinical optical diagnosis and therapy. But it also functions as a natural barrier. The outmost layer of stratum corneum (SC) and the active epidermis limit the permeation of OCAs into tissue to induce optical clearing efficacy. Therefore, skin optical clearing technique has attracted concerns of many researchers. Currently, the technique mainly focused on two aspects. Firstly, the OCAs were directly applied to dermis, including immersion method for in vitro skin [5] or hypodermic injection for in vivo skin [6, 7]. But the direct contact of dermis with OCAs at high concentration can disrupt the normal skin structure and function, yet OCAs at low concentration cannot induce good optical clearing efficacy [8]; secondly, the OCAs were applied to skin epidermis to permeate into dermis. And the skin surface had been disrupted by

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some physical or chemical methods such as sandpaper grinding [9], micro-needle rolling [10], ultrasonic delivery [11], photo irradiation [12], chemical enhancement [13, 14] etc. However, the above methods need some equipment to implement, and what’s more, some even need special technical training. Considering all the above studies, we can conclude that all the existing researches have mainly focused on improving the structure of skin by avoiding or reducing its barrier function. In contrast, investigations about the effect of physical properties of agents on skin optical clearing efficacy have not been reported.

Actually, increasing temperature of drugs has been a common method to improve its permeability in transdermal drug delivery system [15-17]. Literatures have reported that penetrability of aspirin was increased by 15 times when its temperature was increased from 10°C to 40°C. And the absorption of salicylic acid on the abdominal skin of guinea pig can be increased by 5 times when its temperature is increased from 20°C to 30°C [18]. So, do the optical clearing agents have the similar characteristic to skin?

The aim of this study was to investigate the optical clearing efficacy of OCAs at different temperatures. A common used agent——glycerol was chosen in this study. Glycerol at different temperatures (4°C, 25°C, 32°C and 45°C) was applied onto in vitro porcine skin.

2. Materials and methods

2.1. Animal model and agent

In vitro porcine skin was purchased from farmer’s market, and its stratum corneum has been wiped off. After removing the subcutaneous fat tissue, the skin with similar thickness and color were selected and cut into 5×5 cm² pieces as samples ready for use.

Glycerol was an analytical reagent (AR) and was produced by Guangcheng Chemical Reagent Company, Ltd. Tianjin. Before each experiment, glycerol contained in a plastic tube was placed into 4°C refrigerator or 37°C water bath to maintain for at least 2 hours. During the experiment, the tube was also kept in the same place.

2.2. Detection System

In this work, a commercially available spectrophotometer (Lambda 950, PerkinElmer, USA) with a 150 mm integrating sphere setup was utilized to measure the reflectance and transmittance of in vitro skin samples. The sphere has an entrance-port and an exit-port of 1 inch in diameter. The scanning wavelength range was 400-1400 nm with 10 nm interval. Particularly, the data at 630 nm have been attained to quantitatively analysis.

2.3. Experimental Methods

The standby skin samples were divided into four groups and there was 5~7 samples in each group. All the experiments were conducted under room temperature at 23±2°C. Skin samples in each group were treated respectively by glycerol at different temperatures of 4, 25, 32 and 45°C.

Before experiments, each skin sample was sandwiched between two glass slides with dimension of 50×50 mm². Then the thickness of sandwich was measured 4 times by a micrometer from 4 directions to calculate an average as its whole thickness. After that, the
Native transmittance and reflectance spectra were measured using the spectrophotometer with integrating sphere system. And then all skin samples were put on thermostatic plate with temperature of 32°C and applied glycerol on the epidermal side in the central region. After 5 min, the glycerol was wiped off and the sandwich thickness, reflectance spectra and transmittance spectra were repeatedly measured from glycerol treating time of 5 min to 30 min, with 5 min interval. Each time after measurement, glycerol was applied to the epidermal side. At last, the thickness of skin sample was determined by subtracting thickness of two glass slides from the whole thickness.

2.4. Data Analysis

Based on values of reflectance and transmittance as well as the thickness of sample, the reduced scattering coefficient and absorption coefficient was calculated using an inverse adding-doubling (IAD, Scott Prahl, 2007) program. And further, the Penetration depth of photons in skin can be calculated by the following formula [19]:

$$\delta = \frac{1}{\sqrt{3 \mu_s (\mu_a + \mu_s)}}$$  \hspace{1cm} (1)

Here $\mu_a$ and $\mu_s$ are absorption coefficient and reduced scattering coefficient at specific wavelength, respectively.

In the study of organism tissue optical clearing technique, the changes of scattering property and penetration depth are usually used to quantitatively evaluate the effect of optical clearing. In order to avoid influence of tissue absorption, near infrared and infrared light with characteristics of high scattering and low absorption was considered in this study. After glycerol treatment, the relative change of reduced scattering coefficient and penetration depth at 630nm were calculated respectively as follows:

$$\mu_{s-rel} = \frac{\mu_{s,t} - \mu_{s,0}}{\mu_{s,0}}$$  \hspace{1cm} (2)

$$\delta_{rel} = \frac{\delta_t - \delta_0}{\delta_0}$$  \hspace{1cm} (3)

Here $\mu_{s,0}$ and $\delta_0$ represent the original reduced scattering coefficient and original penetration depth of skin, respectively. $\mu_{s,t}$ and $\delta_t$ represent the reduced scattering coefficient and penetration depth after glycerol treatment for t min, respectively. $\mu_{s-rel}$ and $\delta_{rel}$ represent the relative change of reduced scattering coefficient and penetration depth of skin after glycerol treatment.

Finally, in order to compare the significance level (P<0.05) of optical clearing efficiency of glycerol at different temperature, SPSS 13.0 software was utilized for statistically analyzing the relative change of reduced scattering coefficient and penetration depth in different groups.

3. Results and Analysis

3.1. Spectral characteristics changes of in vitro skin before and after glycerol application
In the study, the reflection and transmission spectrum of skin before and after treatment by glycerol at different temperature for different time were measured. However, due to the difference of skin thickness, it makes no sense to compare the absolute values of reflection and transmission. Instead, the reduced scattering coefficient can represent skin optical clearing efficacy more precisely. Because there is little impact for optical clearing on skin absorption in the visible and near infrared wavelength bands, here, Figure 1 shows the typical reflection spectra, transmission spectra and reduced scattering coefficient spectra between 400-1400 nm before and after treatment for 10min, 20min, 30min with glycerol at 4°C, 25°C, 32°C and 45°C.

From Figure 1, it can be seen that treatment of glycerol at different temperature decreases the reflection spectrum and the reduced scattering coefficient spectrum, and increased the transmittance spectrum. The longer treatment by glycerol, the more decreasing of reflection spectrum and the reduced scattering spectrum, and the more increasing of transmission spectrum. As can be seen from the right column, after treated the same time, the greater change of reduced scattering spectrum was induced by glycerol at higher temperature.

3.2. The influence of glycerol at different temperatures on reduced scattering coefficient of in vitro skin
In order to compare the influence of glycerol treatment at different temperatures on reduced scattering coefficient of in vitro skin, Figure 2 shows the relative changes of reduced scattering coefficient at 630nm before and after glycerol treatment for 5-30min. After glycerol application, no matter what the temperature is, the reduced scattering coefficients are all decreased. Glycerol treatment at lower temperature of 4°C and 25°C induces the similar decreasing degree and changing tendency. However, glycerol at higher temperature can decrease the reduced scattering coefficient very quickly. For example, during the first 10 min, the 32°C glycerol treatment decreases the parameter to the same degree as glycerol at lower temperature does, but as the treated time prolongs, the 32°C glycerol treatment decreases the parameter more quickly. Apparently, after the first 5 min, the 45°C glycerol treatment can decrease the parameter significantly, and after 15 min, it can decrease the parameter to the same degree as 4°C or 25°C glycerol does for 30 min.

After 30 min for glycerol treatment, 4 °C and 25 °C glycerol decrease the reduced scattering coefficient by 48.2% and 49.7%, respectively, and there is no significant difference (P=0.786). The 32°C glycerol treatment decreased the coefficient by 61.6% and has significant difference compared with other temperatures (P<0.05). The 45°C glycerol decreased the coefficient by 76.6% and has extreme significant difference (P<0.01).

3.3. The effect of glycerol with different temperature on light penetration depth of in vitro skin
Figure 3 shows the relative change of penetration depth at 630 nm of in vitro skin treated 30 minutes by glycerol with 4, 25, 32 and 45°C. The relative changes induced by 4 and 25°C glycerol are almost the same, increased by 37.9% and 39.5%, respectively, without significant difference (P=0.625). With the increasing of temperature, the penetration depth relatively changed more significantly. Compared with other three temperatures, the penetration depth induced by treatment of 32 and 45°C glycerol increases by 53.3% and 84.1%, respectively,
Figure 1. Typical changes of skin optical properties induced by anhydrous glycerol. Glycerol temperature: 4, 25, 32 and 45 °C for group A, B, C and D; the left, middle and right column represents reflectance, transmittance and reduced scattering coefficient respectively.

and have significant difference ($P<0.05$) and extreme significant difference ($P<0.01$).
Figure 2. Relative reduction of reduced scattering coefficient of in vitro skin treated by glycerol at different temperature

Figure 3. Relative reduction of penetration depth on in vitro skin induced by glycerol with different temperature

4. Discussion
In this work, the influence of OCA’s temperature on optical clearing efficacy of in vitro porcine skin was explored. Glycerol was of four different temperature, including refrigeration temperature of 4°C, room temperature of 25°C, normal skin temperature of 32°C and skin long tolerance temperature of 45°C.

Temperature of OCAs has always been ignored by the previous studies focusing on tissue optical clearing. Actually, OCAs used in the previous studies were either obtained from refrigerator or kept under room temperature which changed with the environment temperature. The different storage temperatures of OCAs may cause different optical efficiency. So it is very important and necessary to invest the influence of glycerol at different temperatures on skin optical clearing efficacy. Though from this study there was no significant difference
between the optical efficacy of glycerol at 4 and 25°C, glycerol at higher temperature could induce skin optical clearing much faster and much better. In this study, skin samples were put on a homothermal plate set at temperature of 32°C. The 4°C glycerol could gain heat from the skin sample after application and then its temperature may increase, causing the same optical clearing efficacy as 25°C glycerol did. The 32°C glycerol had the same temperature as normal skin had, and the force making it penetrate from epidermis deep into dermis was mainly the concentration gradient and molecule diffusion. As the temperature of glycerol increased to 45°C, the thermal motion of glycerol molecule was more intense and the diffusion rate was increased, making it much easier to diffuse deep into dermis. On the other side, increasing of glycerol temperature would decrease glycerol’s viscosity. As has been reported in the literature [18] about relations between glycerol’s viscosity and its temperature, we could calculate quantitatively that when the temperature increased from 25°C to 32°C or even 45°C, its viscosity would decrease from 0.91 Pa.s to 0.51 Pa.s or even 0.2 Pa.s. The decreasing in glycerol’s viscosity would facilitate its fluidity in the skin, thus increasing the diffusion rate of glycerol molecule.

From both molecule-kinetic theory and glycerol’s viscosity, we can conclude that increasing of glycerol’s temperature would increase its perfusion rate and facilitate glycerol perfusion into dermis, which make more glycerol molecule penetrate into dermis to induce optical clearing. The high permeability of glycerol would induce tissue dehydration, that is the tissue fluid with lower index of refraction would be replaced by glycerol with higher index, and make the refractory index of different components in the skin matched, which could decrease light scattering and achieve optical clearing efficacy. Thus, increasing the temperature of glycerol could enhance skin optical clearing significantly. However, there is limitation for skin to endure higher temperature for long time, glycerol with temperature higher than 45°C should not be applied to skin for long time. For this consideration, it may also effective to enhancing skin optical clearing by increasing skin’s temperature or exposing skin to a high-temperature in super-short time [20]. In addition, investigations on the combination of this work and other physical or chemical methods to enhance skin optical clearing are also deserve further study.

5. Conclusion
From this study, it can be concluded that glycerol at different temperatures has different effect on skin optical clearing. Compared with normal skin temperature, glycerol at lower temperature has almost the same effect on enhancing optical clearing, and glycerol at higher temperature could induce skin optical skin clearing much faster and more significant, especially, the higher the temperature, the better and faster the enhancing optical clearing efficacy. This study provides an easy and effective way to enhance skin optical clearing.

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