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Ear skin optical clearing for improving blood flow imaging

Optisches Clearing der Ohrhaut zur verbesserten Bildgebung des Blutflusses

Abstract

Background and objective: Various optical imaging techniques have shown a great potential for monitoring angiogenesis, development of blood vessels, and even tumor transfer, but they suffer from the limited imaging depth in tissue. Although the mouse ear provides an available window, the residual scattering of ear skin still influences the imaging quality. The proposed tissue optical clearing technique presents a new opportunity to decrease the scattering of skin, and enhance the imaging contrast or imaging depth of optical methods. The purpose of this study is to develop an innovative ear skin optical clearing agent (ESOCA) for improving the transparency of the mouse ear.

Materials and methods: The ESOCA was topically applied on the ear skin of BALB/c mice *in vivo* for 5 and 10 min, respectively. Then the transmittance spectra of mice ear were measured with an optical fiber spectrometer system, and the cutaneous blood vessels and blood flow was monitored by the laser speckle contrast imaging (LSCI) technique. As a control, the measurements were also performed before application of the ESOCA. In addition, the contrast-to-noise ratio (CNR) values of speckle contrast images were calculated to evaluate the resolving ability to blood flow.

Results: The transmittance of mice ear was enhanced by $111.0 \pm 8.2\%$ at 633 nm after application of ESOCA. The cutaneous blood vessels and blood flow could be distinguished more clearly with LSCI technique. In addition, the calculated CNR values of speckle contrast images showed a great enhancement compared with the initial (control) values.

Conclusion: In summary, topical application of an innovative ESOCA permits the vessel structure and flow distribution information of cutaneous blood vessels to be imaged by LSCI with higher contrast, which will be significant for tumor studies in the future.

Keywords: ear skin optical clearing; cutaneous blood flow imaging; laser speckle contrast imaging technique; contrast-to-noise ratio.

Zusammenfassung

Hintergrund und Ziel: Verschiedene optische bildgebende Verfahren haben ein großes Potenzial für die Überwachung der Angiogenese, der Entwicklung von Blutgefäßen und sogar der Tumorausbreitung gezeigt, leiden aber unter der eingeschränkten Bildtiefe im Gewebe. Das Mausohr bietet modellhaft betrachtet ein optisches Fenster, jedoch wird die Bildqualität durch die restliche Streuung der Ohrhaut beeinflusst. Die vorgeschlagene gewebeoptische Clearing-Technik stellt eine neue Möglichkeit dar, die Streuung der Haut zu verringern und Bildkontrast und Bildtiefe optischer Verfahren zu verbessern. Das Ziel dieser Studie ist es, ein innovatives Hautclearing-Agent (ear skin optical clearing agent, ESOCA) zur Verbesserung der Transparenz des Mausohrs zu entwickeln.

Materialien und Methoden: Das ESOCA wurde *in vivo* für jeweils 5 und 10 min topisch auf die Ohrhaut von BALB/c-Mäusen aufgetragen. Dann wurden am Mausohr die Transmissionsspektren mit einem optischen Faserspektrometer gemessen und die kutanen Blutgefäße und der Blutfluss mittels Laser-Speckle-Kontrastdarstellung (laser speckle contrast imaging, LSCI) überwacht. Als Kontrolle wurden die Messungen auch vor dem Aufbringen des ESOCA durchgeführt. Zusätzlich wurde das Kontrast-Rausch-Verhältnis (contrast-to-noise ratio, CNR) der Speckle-Kontrastbilder berechnet, um das Auflösungsvermögen in der Darstellung des Blutflusses zu evaluieren.

Ergebnisse: Die Durchlässigkeit der Ohrhaut von Mäusen wurde nach Anwendung des ESOCA und bei einer Wellenlänge von $\lambda=633$ nm um $111,0 \pm 8,2\%$ erhöht. Die kutanen Blutgefäße und der Blutfluss konnten mit der LSCI-Technik deutlich unterschieden werden. Darüber hinaus zeigten die berechneten CNR-Werte eine große Verbesserung im Vergleich zu den eingangs durchgeführten Kontrollmessungen (ohne ESOCA).

Fazit: Die topische Anwendung des innovativen ESOCA erlaubt eine kontrastreichere Darstellung der Gefäßstruktur und der Strömungsverteilung von kutanen Blutgefäßen mittels LSCI-Technik. Dies könnte zukünftig von entscheidender Bedeutung für Tumorstudien sein.

Schlüsselwörter: optisches Clearing der Ohrhaut; Bildgebung der Hautdurchblutung; Laser-Speckle-Kontrastdarstellung; Kontrast-Rausch-Verhältnis.

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1 Introduction

Understanding of the gene expression, physiological function and treatment efficacy is crucial for tumor study [1]. Thus, the ability to monitor tumor growth, transfer, angiogenesis and blood vessel recruitment is of considerable importance [2]. Various imaging techniques have been applied [1–8], among which, some optical methods have shown substantial advantages over others, benefiting from their high spatiotemporal resolution [1, 2, 4–8]. However, the penetration depth of light is limited by the strong scattering of turbid tissue, and previous studies were mostly based on transparent tissue models, such as the dorsal skin chamber [1, 4, 9], cranial window [1], and chick chorioallantoic membrane [9], etc. The specific microenvironment and semi-transparent property also permits the mouse ear to become an available window for continuously studying the angiogenic activities of tumors [2]. Unfortunately, the residual scattering of the ear skin still influences the optical imaging quality.

Traditionally, immersion in cedar-wood oil or Vaseline oil has been applied in classical microscopy or capillaroscopy to improve light penetration [10]. In 1997, Tuchin et al. proposed the tissue optical control technique [11], which can enhance the light penetration depth by immersion of tissues into optical clearing agents (OCAs). This technique is also called tissue optical clearing technique [12–17]. Among investigations on optical clearing of tissues, skin optical clearing has attracted more attention [18–21]. For instance, by topically applying an optical clearing agent on dorsal skin of rat *in vivo*, information about the dermal blood vessels and blood flow could be acquired non-invasively with laser speckle contrast imaging (LSCI) technique [18, 19]. However, as yet the optical clearing of ear skin has not been reported.

The purpose of this study is to develop an ear skin optical clearing agent (ESOCA) to make the ear skin more transparent. To quantitatively demonstrate the effectiveness of the agent, an optical fiber spectrometer was used to measure the changes in transmittance of mouse ear before and after topical application of ESOCA. Furthermore, the LSCI technique was applied to monitor the blood vessel structure and blood flow distribution. The contrast-to-noise ratio (CNR) of speckle contrast images was also calculated to evaluate the improvement of imaging quality and the resolving ability to the blood flow.

2 Materials and methods

2.1 Chemical agents and animal preparation

The innovative ESOCA used in this study is composed of several biocompatible agents, whose main components include polyethylene glycol (PEG-400), fructose and thiazone. The exact composition and fabrication cannot be described in detail as for the ESOCA is actually a patent pending.

Animal care and experimental procedures were approved by the Experimental Animal Management Ordinance of Hubei Province, China. Male BALB/c mice ($n=6$, 25 ± 5 g, 8 weeks old) were supplied by Wuhan University Center for Animal Experiment, Wuhan, China and fed under specific pathogen free feeding conditions.

Mice were anesthetized with the mixture of 2% α -chloralose and 10% urethane (8 ml/kg) via intraperitoneal injection. The hair on the ears of the mice was removed with depilatory cream (sensitive hair removal cream; M/s Veet), so that the animal model was well prepared for the trial.

2.2 Optical fiber spectrometer system for measuring transmittance of mouse ear

In this study, an optical fiber spectrometer system (USB-4000; M/s Ocean Optics, USA) was applied to measure the transmittance of the mouse ear. Figure 1 shows the schematics of this system. A reflection and transmission stage (Stage-RTL-T; M/s Ocean Optics, USA), containing a sample holder and two fiber holders, was used to fix the mouse ear and the optical fibers. A calibrated tungsten halogen light source (HL-2000-CAL; M/s Ocean Optics, USA) coupled with an optical fiber (QP600-2-VIS/NIR; M/s Ocean Optics, USA) was used to illuminate the region of interest of the mouse ear, and another optical fiber of the same type coupled with the spectrometer system was used

Figure 1 Schematic of optical fiber spectrometer system.

to collect the transmitted light in the spectral range of 400–900 nm. Here, two collimating lenses were coupled with the two optical fibers, respectively, so as to measure the collimated transmittance. The core diameter of the optical fiber is $600 \pm 10 \mu\text{m}$.

After the animal model had been prepared, the initial transmittance spectrum was obtained from the region of interest of the ear. Then the ESOCA was topically applied on the skin of the mouse's ear *in vivo*, and the transmittance spectrum was measured after 5 min and 10 min, respectively. Before each measurement, the ESOCA was gently removed from the ear skin so as to reduce the specular reflection, and applied once again after measurement.

2.3 Laser speckle contrast imaging technique for assessing cutaneous blood flow

2.3.1 Laser speckle contrast imaging system

The cutaneous blood vessels and blood flow were monitored using the LSCI technique (Figure 2). A helium neon laser ($\lambda=632.8 \text{ nm}$, 3 mW) was used to illuminate the region of interest with a beam expanded through a collimating lens. The illuminated regions were then imaged through a stereo microscope (SZ61TR; M/s Olympus, Japan) onto a CCD camera (Pixelfly USB; M/s PCO Computer, Germany). The exposure time used here was 5 ms and if a white-light was used instead of the laser source, white-light images could be obtained.

2.3.2 Experimental protocol

The prepared mouse model was placed on the experimental platform, and the initial white-light and raw speckle images of the area of interest were obtained. Then, the ESOCA was topically applied on the skin of the mouse

Figure 2 Schematic illustration of laser speckle contrast image system.

ear *in vivo* and a sequence of white-light and raw speckle images were recorded after a 5 min and 10 min interval after treatment of ESOCA, respectively. Then the laser speckle temporal contrast analysis method was applied to obtain two-dimensional blood flow distribution images of cutaneous blood vessels, which has been reported to be highly effective for suppressing the influence of static speckle from a surrounding scattering medium [22].

2.3.3 Data analysis

The CNR was used to evaluate the improvement in the ability to resolve the blood vessels before and after application of ESOCA on ear skin. The definition of CNR is as follows [23]:

$$\text{CNR} = \frac{TC_{\text{back}} - TC_{\text{vessel}}}{\sqrt{f_{\text{vessel}} \sigma_{\text{vessel}}^2 + f_{\text{back}} \sigma_{\text{back}}^2}} \quad (1)$$

where TC_{vessel} and TC_{back} denote the mean values of temporal contrast constituting the blood flow and background, respectively. σ_{vessel}^2 and σ_{back}^2 denote the variance in the values of the temporal contrast constituting the blood flow and the background, respectively. f_{vessel} and f_{back} are the fractions of the total number of pixels selected in the temporal contrast image classified as blood flow and background, respectively.

3 Results

3.1 Changes in transmittance properties of mouse ear

The reflectance spectrum is commonly used to evaluate the optical clearing efficacy of skin *in vivo* [12, 20, 24].

Figure 3 Changes in transmittance of mouse ear before (control value), and 5 min and 10 min after application of ESOCA, respectively.

In fact collimated transmittance is more direct, especially for the thin mouse ear. Therefore, changes in collimated transmittance of the mouse ear were measured in this study to quantitatively evaluate the optical clearing effect of ESOCA. A typical result is shown in Figure 3. It can be found that after treatment of ESOCA for 5 min, the transmittance spectrum increases significantly. However, changes in the transmittance spectrum were very small during the 5 min to 10 min time period. In addition, the

relative change at 633 nm was also calculated and it was apparent that compared to the initial (control) value, the transmittance increases by $111.0 \pm 8.2\%$ after topical application of ESOCA for 10 min.

3.2 ESOCA-induced optical clearing of mouse ear skin *in vivo*

The above results demonstrate that application of ESOCA can significantly enhance the transmittance of mouse ear. Furthermore the blood vessel structure and flow distribution was monitored with LSCI technique, and typical results are shown in Figure 4. Figure 4A–C shows the white-light images before, 5 min and 10 min after application of ESOCA, respectively; Figure 4D–F shows the corresponding laser speckle temporal contrast images (LSTCI); Figure 4G–I shows the profiles of temporal contrast values along the horizontal line in Figure 4D–F, respectively.

It can be seen that initially, the ear skin is semitransparent and the structure and flow information of some of the small blood vessels are not visible. After topical application of ESOCA on the ear skin for 5 min, the mouse ear becomes more transparent and even the small branches of cutaneous blood vessels can be clearly observed in the

Figure 4 (A–C) White-light and (D–F) laser speckle temporal contrast images before (control), and 5 min and 10 min after application of ESOCA on to the mouse ear, respectively. The numbers 1–9 in (D–F) indicate the blood vessels of interest. (G–I) Profiles of speckle temporal contrast values along the horizontal line in (D–F). The arrows in (G–I) indicate the location of vessel #3.

Blood vessels	Initial measurement	Measurement after topical application of ESOCA	
		5 min	10 min
Vessel 1			
Diameter $\pm$ SD (μ m)	37.70 $\pm$ 2.20	37.98 $\pm$ 1.14	37.41 $\pm$ 2.16
<i>p</i> -value	–	0.80	0.80
Vessel 2			
Diameter $\pm$ SD (μ m)	69.23 $\pm$ 2.23	69.95 $\pm$ 3.11	69.80 $\pm$ 2.85
<i>p</i> -value	–	0.66	0.72
Vessel 4			
Diameter $\pm$ SD (μ m)	50.60 $\pm$ 2.69	51.17 $\pm$ 2.81	50.45 $\pm$ 3.29
<i>p</i> -value	–	0.74	0.93
Vessel 5			
Diameter $\pm$ SD (μ m)	22.65 $\pm$ 3.25	23.94 $\pm$ 1.27	22.65 $\pm$ 2.01
<i>p</i> -value	–	0.35	1.00
Vessel 6			
Diameter $\pm$ SD (μ m)	27.09 $\pm$ 1.61	27.66 $\pm$ 1.48	28.09 $\pm$ 1.04
<i>p</i> -value	–	0.49	0.23
Vessel 7			
Diameter $\pm$ SD (μ m)	35.83 $\pm$ 1.60	36.98 $\pm$ 1.09	36.12 $\pm$ 2.18
<i>p</i> -value	–	0.26	0.77

Table 1 Measurement of diameters of some typical blood vessels shown in Figure 4.

white-light images. The LSTCI show that it is still difficult to distinguish the blood flow information of vessel #3 at initial state. After treatment with ESOCA for 10 min, the mouse ear becomes entirely transparent and both the structure and flow distribution information of all the blood vessels can be detected with higher contrast. Thus it can be concluded that smaller branch blood vessels can be distinguished and no blood flow is blocked after application of ESOCA.

In order to quantitatively evaluate the effect of ESOCA on the cutaneous blood vessels, the diameter of some typical vessels in Figure 4D–F is calculated. The results are summarized in Table 1. In addition, the statistical analyses with one-factor analysis of variance were carried out and the least significant difference test was employed to inspect the differences in vessel diameters between 5, 10 min after ESOCA application and initial measurements, respectively. It can be found that after topical application of ESOCA on ear skin, the diameters of cutaneous blood vessels are essentially unchanged compared with the initial measurements of diameter. There are also no significant changes in vessel diameters between 5 min, 10 min and initial ($p > 0.05$). That means, the ESOCA has no obvious side effects on cutaneous blood vessels.

For further comparison, the profiles of speckle temporal contrast values were also plotted along the horizontal line in the speckle images. In the profiles of temporal contrast values, each valley indicates the blood flow of a cutaneous blood vessel that can be resolved. It can be

found that initially, the valley corresponding to the vessel #3 cannot be distinguished from the surrounding background (as the arrow in Figure 4G indicates), and others are available to be detected with low contrast. At 5 min after application of ESOCA, the temporal contrast values of the background area increase, while the temporal contrast values of the blood flow area still remain lower values (Figure 4H). These changes allow the valleys to be more easily distinguished. The valley corresponding to the vessel #3 begin to become more obvious (Figure 4E), but it is still difficult to distinguish it (as the arrow in Figure 4H indicates). After topical application of ESOCA for 10 min, the valley corresponding to the vessel #3 can be detected clearly.

3.3 Changes in CNRs of speckle temporal contrast images

To further quantitatively evaluate the improvement in the ability to resolve blood vessels, the CNRs of speckle temporal contrast images were calculated and a typical result is shown in Figure 5. It can be found that for all nine blood vessels shown in Figure 4D–F, the CNRs of speckle contrast images increase considerably at 10 min after application of ESOCA compared with that at initial (control), which indicates that optical clearing of skin can enhance the resolving ability to blood flow effectively. In addition, the mean improvement in CNRs before and

Figure 5 Typical CNRs of the speckle temporal contrast images before (control value) and 10 min after topical application of ESOCA. The vessel indexes 1–9 indicate the vessels 1–9 shown in Figure 4D (control) and F (ESOCA-10 min), respectively.

10 min after application of ESOCA was also calculated, and it can be found that the CNRs of speckle contrast images are enhanced by $128 \pm 21\%$ and $49 \pm 7\%$ when the diameters of blood vessels are between $25 \mu\text{m}$ and $30 \mu\text{m}$ or $50 \mu\text{m}$ and $80 \mu\text{m}$, respectively.

4 Discussion

The above results indicate that topical application of ESOCA on ear skin *in vivo* can enhance the transmittance of mouse ear and improve the imaging contrast of cutaneous blood vessels and blood flow. Compared with the intact case, the CNRs of speckle contrast images increased by $128 \pm 21\%$ and $49 \pm 7\%$ after application of ESOCA when the diameter of blood vessels are between $25 \mu\text{m}$ and $30 \mu\text{m}$, or $50 \mu\text{m}$ and $80 \mu\text{m}$, respectively. In this study, fructose was added as a component to the formula of our optical clearing agents. Fructose is hygroscopic which makes it quicker to absorb water and slower to release it to the environment. The high osmotic pressure of fructose also induces a faster water flux from tissue [25]. Hence, addition of fructose to the mixture of PEG-400 and thiazone produced an excellent optical clearing effect.

In the previous studies, various OCAs have been introduced to make skin [18, 19], dura mater [26] and skull [17] more transparent. Even though each component of OCAs is biocompatible, direct action of OCAs with higher concentration on the blood vessels might induce decrease of blood flow velocity, blockage or even impairment of blood vessels [27–29]. For instance, Vargas et al. [27] added 100% and 75% glycerol on the subdermis of hamster and found that the blood flow was blocked in the venules. Galanzha et al. [28] found that after 75% glycerol or 20–40% glucose were added to the mesentery of rat, the microvessels were

blocked to different extents. Cheng et al. [26] noticed that application of glycerol on rabbit dura mater would induce changes in blood flow velocity. Zhu et al. [29] found that glycerol could induce serious short-term effects on blood vessels in chick chorioallantoic membrane, and glucose could also induce serious long-term effects. By decreasing the concentration, or the dose of the agents that reached the vessels, the side effects were reduced [29]. Notably, Zhu et al. [18] also found that topical application of a mixture of PEG-400 and thiazone on dorsal skin of rat *in vivo* allowed the dermal blood vessels to be imaged by LSCI technique non-invasively, and the blood flow were not affected by the mixture solution. Recently, they repeatedly applied the mixture solution on rat skin *in vivo*, and found that there were no long-term effects on the skin and body [19]. In this work, in addition to PEG-400 and thiazone, fructose was added to the formula of ESOCA. Even though the short-term effects of ESOCA on blood vessels and blood flow were not observed, more detailed investigations on the long-term influence of ESOCA need to be performed in the future.

In this work, the ability to resolve blood flow is greatly improved by topical application of ESOCA on ear skin of mouse *in vivo*. For intact ear skin, the existence of strong background noise caused by skin scattering interferes with the resolution of the blood flow, such as the small fluctuations indicated by the arrow in Figure 4G. Application of ESOCA can reduce skin scattering and therefore helps to diminish the interference caused by background noise. However, a 5 min-application of ESOCA does not make the skin of the ear become entirely transparent, and the residual scattering still interferes with the measurement of blood flow by LSCI technique, making it difficult to distinguish the blood flow information of small branch vessels from the background noise. This is why the blood vessel #3 can be observed clearly in the white-light images, but the blood flow information is still difficult to be detected in the speckle temporal contrast images (compare Figure 4B and E). A 10-min application of ESOCA can make the ear skin become entirely transparent, which allows the LSCI technique to assess the blood vessel structure and blood flow distribution information of mouse ear with high CNR.

The mouse ear is mainly composed of skin and cartilage with thin layers of subcutaneous tissue, and there are numerous mast cells near the small vessels in dermis and subcutaneous tissue [2]. Mast cells can contribute to angiogenesis and wound healing since they are able to produce growth-promoting and pro-inflammatory mediators [2]. Consequently, the mouse ear is not only a good model to investigate tumor angiogenesis, but also a suitable model for immunity studies. In the studies of

tumor and inflammation, acquisition of the quantitative information about the number and flow characteristics of tumor cells and leukocyte populations is of considerable importance [30–33]. However, the scattering of the skin increases the difficulty to track these cells. Fortunately, the optical clearing agent proposed in this work can reduce the skin scattering and bring new opportunities for monitoring tumor cell and leukocyte populations. Further studies will be carried out in the future.

5 Conclusion

In summary, topical application of an innovative skin optical clearing agent, ESOCA, on the ear skin of mouse

in vivo can greatly improve the imaging of the structure and flow distribution of cutaneous blood vessels with LSCL. The ability to resolve blood flow can also be enhanced by treatment with ESOCA. If the optical clearing method is applied for tumor and inflammation studies in the future, we believe that exciting new achievements could be possible.

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