Comparison of Cortical and Cutaneous Vascular Hemodynamic Changes in Hypoxia by Using *in Vivo* Skull and Skin Optical Clearing Techniques

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***Abstract*—Hypoxia occurs in various pathophysiological conditions. Especially, brain can be seriously affected by the oxygen deficiency. However, challenges exist in optically monitoring cerebral hemodynamics with high resolution. As an easily-detected vessel bed, peripheral skin is a potential target for predicting cerebrovascular hemodynamic changes. However, the similarities and differences between cerebral and cutaneous hemodynamics during hypoxia are still unclear. One of the main reasons is that optical imaging techniques are fundamentally depth/resolution-limited due to high scattering properties of turbid tissue. Fortunately, *in vivo* tissue optical clearing techniques can efficiently overcome these problems, and avoid the side-effects of surgical windows. In this work, we simultaneously monitor the changes in cortical and cutaneous microvascular blood oxygen and blood flow under the assistance of *in vivo* skull and skin optical clearing techniques, and quantitatively compared the differences between cerebral and cutaneous arteriovenous functional responses to the hypoxic stimulus. The results indicated that the variation tendency of blood oxygen response might be more similar, and cutaneous vascular blood oxygen response has the potential to serve as an accessible indicator for revealing cerebrovascular dysfunction. Moreover, it provides a feasible approach to realize visualization of *in vivo* monitoring cerebral and cutaneous microvascular reactivity with minimal invasiveness.**

***Index Terms*—Hyperspectral imaging, hypoxia, laser speckle contrast imaging, skin optical clearing, skull optical clearing.**

I. Introduction

TISSUE hypoxia plays an important role in the pathophysiology of various human disorders, e.g., ischemic cardiovascular disease, stroke, chronic lung disease, acute skin wounds and cancer [1]–[4]. As one of the most important organs, the brain needs continuous and adequate supply of oxygen to maintain its structural and functional integrity, and even moderate reduction in oxygen delivery can cause serious brain dysfunction [5], [6]. Also, the hypoxia has an effect on other tissues and organs including peripheral skin [7]. There have been some researches investigate the changes in blood flow or blood oxygen of brain and skin by using various techniques, i.e., Duong *et al.* employed MRI to monitor the cerebral blood flow velocity changes induced by hypoxia [8]; Palmer *et al.* used spectral imaging to investigate the cutaneous blood oxygen changes around the tumor [9]. However, the imaging resolutions of these approaches have been greatly limited.

Nowadays, optical imaging techniques have received more and more attentions in studying microvascular functions thanks to the advantage in the high resolution, such as optical coherence tomography (OCT), photoacoustic tomography, multiphoton microscopy, laser speckle contrast imaging (LSCI) and hyperspectral imaging technique (HSI). However, the high scattering properties of skin and skull seriously limit the imaging depth and resolution. Fortunately, the optical clearing imaging windows established by *in vivo* optical clearing techniques provide the powerful tools to effectively improve both imaging depth and contrast in various optical imaging modalities without applying general imaging windows based on the complex surgeries. The recently-developed skin optical clearing technique can greatly enhance the imaging qualities in confocal microscopy [10], photoacoustic tomography [11], [12], OCT [13]–[15], LSCI [16] and HSI [17], allowing to observe the immunocyte movement [10], as well as the structural and functional changes in cutaneous vessels [18], [19]. In addition, the skull optical clearing technique permits us to monitor the neuronal synapse growth in young mice [20], and the cerebrovascular dynamics under physiopathological conditions [21]–[23].

Compared with the brain, peripheral skin is a more accessible and generalized vascular bed for clinical diagnosis. Some studies have identified that the peripheral skin has a great potential to be a sufficient biomarker for predicting cardiovascular diseases [24], [25] and diabetic retinopathy [26], as well as a prognostic indicator for evaluating the effect of drugs on the microcirculation [27], [28]. Besides, some diseases and stimuli can cause similar changes in both skin and brain vessels [29]. Our recent work suggests that skin vascular blood oxygen response to the vasomotor test has a good potential to serve as an assessment

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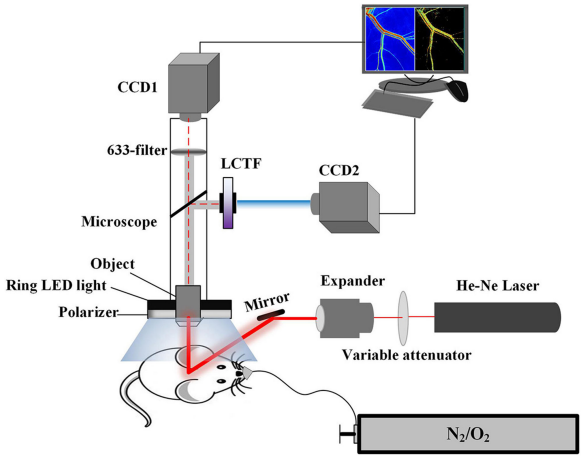

Fig. 1. Schematic of the imaging system.

indicator to predict the cerebrovascular dysfunction caused by type 1 diabetes [30]. As an easily accessible vascular bed, the peripheral skin will be a fine detection target for early warning to some brain diseases. At present, researches about the hemodynamic changes induced by hypoxia only focus on brain or skin separately, neglecting to establish their relations [8], [9]. Thus, the completion of comparative study between brain and skin microvascular functional changes induced by hypoxia can both reveal their relations and differences, and further illustrate whether skin vascular functional changes could be a potential indicator in diagnosing cerebrovascular dysfunction.

In this work, with the help of *in vivo* skull and skin optical clearing techniques, the dual-mode imaging system of HSI and LSCI was employed to simultaneously monitor the changes in blood flow and blood oxygen of cerebral and cutaneous microvessels with hypoxic stimulus. In addition, the rates of relative changes in blood oxygen and blood flow velocity for cerebral and cutaneous arteriovenous vessels were quantitatively analyzed during hypoxic challenge and recovery.

II. Materials and Methods

A. Monitoring Hemodynamic Changes in Vascular Blood Flow and Blood Oxygen

Fig. 1 shows the schematic of imaging system. In order to simultaneously monitor vascular blood flow and blood oxygen, we combined the LSCI and HSI. This system mainly consists of two charge coupled device (CCD) cameras (Pixelfly USB, PCO Company, Germany), a liquid crystal tunable filter (LCTF, CRi Varispec VIS, Perkin Elmer, USA), a stereo microscopy (SZ61TR, Olympus, Japan), a ring-like LED light with a polarizer, a filter (633 ± 5 nm) and a He-Ne laser beam ($\lambda = 632.8$ nm, 3 mW) expanded by a collimating lens used to illuminate the areas of interest. The bandwidth of LCTF is 7 nm. A certified reflectance standard (SRS-99-020, Labsphere, USA) was used to acquire the standard hyperspectral images. All the hyperspectral images were acquired from 500 nm to 620 nm with a step size of 10 nm.

We used the algorithm developed previously to obtain blood oxygen saturation (SO_2) based on the hyperspectral dataset acquired by CCD2 [17], by which the arteries and veins can be well distinguished. The corresponding vascular blood flow can be obtained based on the recorded raw speckle images (The data was acquired by CCD1) by using laser speckle temporal contrast analysis method [31]. At 540 nm, both arteries and veins can be clearly observed due to the strong absorption of oxy-hemoglobin and deoxyhemoglobin. But at the wavelength of 620 nm, the absorption of deoxyhemoglobin is much higher than that of oxy-hemoglobin, thus, only the vein with rich deoxyhemoglobin can be clearly identified in the image. By this means, the artery and vein can be well distinguished.

B. Animal Preparation

Experimental procedures and animal care were approved by the Experimental Animal Management Ordinance of Hubei Province, P. R. China. Male BALB/c mice (25 ± 2g, 8 weeks old, $n = 10$) were supplied by Wuhan University Center for Animal Experiment (Wuhan, P. R. China) and fed under specific pathogen free (SPF) level of feeding condition. All experimental procedures were performed according to animal experiment guidelines of the Experimental Animal Management Ordinance of Hubei Province, P. R. China, and the guidelines from the Huazhong University of Science and Technology, which have been approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology.

C. In Vivo Skull and Skin Optical Clearing Techniques

Here, we used the newly-developed *in vivo* skin optical clearing technique to establish the optical clearing skin window for monitoring the subcutaneous vessels with the high resolution. After the hair removal, skin optical clearing agent (skin-OCA) was topically applied on the region of interest on dorsal skin, which was mixed with polyethylene glycol, thiazone and sucrose (67.1% wt/wt) at a volume ratio of 9:1:10 according to our previous work [32]. 15 min later, the treated skin became optically transparent, and cutaneous vessels can be well observed in this work [32].

The recently-developed *in vivo* skull optical clearing technique allow us to noninvasively monitor the cortical vessels through the intact skull. Firstly, the head hair was removed and an incision was made on the skin, then we used the skull optical clearing agent (skull-OCA) to establish the optical clearing skull window. About 15 min later, the exposed skull became optically transparent. The details about the preparation of *in vivo* skull optical clearing agent can refer to our recent work [22].

D. Hypoxia Experiment

At the normal state, mouse breathed the mixed gas (20% oxygen/78.5% nitrogen) under 1.5% isoflurane prior to the start of hypoxia phase. Then, the mouse was switched from the oxygen enriched air to another mixed gas (10% oxygen/ 88.5% nitrogen) with 1.5% isoflurane, and the hypoxia stimulus was maintained for 3 mins followed by a 4 min recovery period in

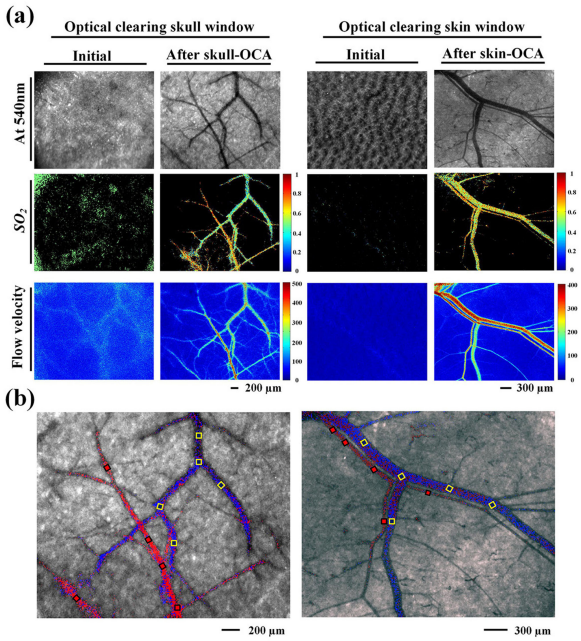

Fig. 2. Cortical/subcutaneous microvessels images before and after optical clearing treatment. (a) Left: the image at 540 nm, SO_2 and blood velocity maps of cortical vessels before and after skull optical clearing treatment. Right: the white-light, SO_2 and blood velocity maps of subcutaneous vessels before and after skin optical clearing treatment. (b) The maps of cerebral and cutaneous vessels extraction, respectively. The vessels colored by red are arteries, and the blue ones represent veins. The black and yellow rectangular regions were selected for SO_2 and blood flow velocity analysis.

which the oxygen and nitrogen were switched back to the 20% oxygen/78.5% nitrogen. And the data were recorded for 3 min at 10 seconds interval before hypoxia.

III. Results

A. Visualization of Cortical and Cutaneous Microvessels By Using in Vivo Optical Clearing Techniques

Here, the dual-mode system of HSI and LSCI was employed to simultaneously monitor the SO_2 and blood flow velocity changes in cortical and cutaneous microvessels. Fig. 2 shows the cortical and subcutaneous microvessels images before and after skull and skin optical clearing. Due to the high scattering properties of the intact skull and skin, it is difficult to distinguish cortical and cutaneous microvessels through skull and skin at 540 nm (Fig. 2(a)). Whereas, the cortical and cutaneous microvessels were clearly observed after the optical clearing skull and skin windows establishment, and the imaging contrast was greatly improved. Thus, the cortical and cutaneous microvascular SO_2 and blood flow velocity maps could be obtained with the high resolution (Fig. 2(a)).

Moreover, based on the differences between the extinction coefficients of oxy- and deoxy-hemoglobin, the arteries and veins were extracted and colored by red and blue, respectively (Fig. 2(b)). The result shows that *in vivo* optical clearing techniques can improve the imaging quality dramatically, which permits to simultaneously monitor the cortical and cutaneous

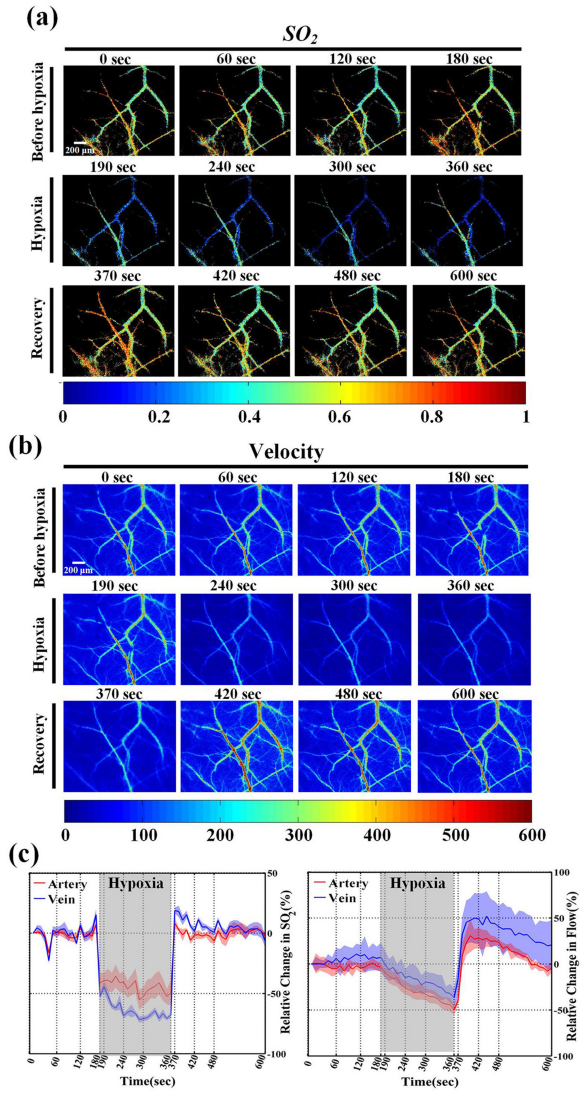

Fig. 3. Monitoring SO_2 and blood flow velocity changes in cerebral microvessels. (a) The pseudo-colored images show the spatiotemporal changes of SO_2 maps in the cerebral microvessels at initial, hypoxia and recovery phases through optical clearing skull window. (b) The pseudo- colored images show the spatiotemporal changes of blood flow velocity maps in the cerebral microvessels at initial, hypoxia and recovery phases. (c) The statistic graphs show relative changes in SO_2 and blood flow velocity of the arteries and veins at different states. The gray shade area indicates the hypoxic stimulus phase. And the curves and the width of shadows are mean and standard deviation, respectively. (Scale bar: 200 μm).

microvascular SO_2 and blood flow velocity changes by HSI and LSCI with high spatial resolutions.

B. Monitoring Cortical Microvascular SO_2 and Blood Flow Velocity Changes to Hypoxia

Further, we simultaneously monitored the cortical microvascular SO_2 and blood flow responses to the hypoxic stimulus. Fig. 3 shows the dynamic changes in cortical microvascular SO_2 and blood flow velocity at initial, hypoxia and recovery phases. When the hypoxic stimulus was implemented, both SO_2 and blood flow velocity of cortical microvessels decreased as shown in Fig. 3(a) and (b). And after hypoxia stimulus, cortical

microvascular SO_2 and blood flow velocity could be almost back to the original level.

In addition, we quantitatively analyzed the arteriovenous vascular relative changes in both SO_2 and blood flow velocity via the black and yellow rectangular regions as indicated in Fig. 2(b). Results showed that the SO_2 had a sharp decline when hypoxia stimulus occurred. Compared with the initial, the arteries decreased by ∼40–50%, and the veins reduced by ∼50–70% at the duration of hypoxia. For blood flow velocity, during the hypoxia course, it steadily decreased, and the falling range was slower than that of SO_2. During the recovery, the SO_2 quickly recovered and was nearly comparable with that in initial. But the blood flow velocity increased to a much higher level and then gradually declined (Fig. 3(c)). The result indicated that hypoxic stimulus not only caused changes in the microvascular blood oxygen but also in the blood flow, nevertheless, there are some differences in their trends.

C. Monitoring Cutaneous Microvascular SO_2 and Blood Flow Velocity Changes to Hypoxia

Additionally, the changes of cutaneous microvascular SO_2 and blood flow induced by hypoxia were also monitored with the help of *in vivo* skin clearing technique as shown in Fig. 4. Compared with the response of cerebral microvessels, similarly, the SO_2 and blood flow velocity of arteries and veins in cutaneous microvessels obviously decreased during hypoxic phase. And during recovery, the SO_2 and blood flow velocity of both arteries and veins increased (Fig. 4(a) and (b)). Further, we quantitatively analyzed the arteriovenous vascular relative changes of SO_2 and blood flow velocity via the selected regions as indicated in Fig. 2(b). Results showed that the SO_2 of the arteries and veins quickly decreased by ∼40–50% at the duration of hypoxia compared with the initial. And during the recovery, the arteriovenous vascular SO_2 steadily became back to the normal state. As for blood flow velocity, hypoxia caused a small reduction. The blood flow velocity decreased by ∼13% in veins and ∼20% in arteries at the end of hypoxia, and about 1 min after recovery, the blood flow was nearly back to the initial (Fig. 4(c)). The results showed that the skin microvascular blood oxygen response was similar with the cortical microvessels under the hypoxic stimulus, but there were some differences between skin and brain microvessels in the trend of blood flow.

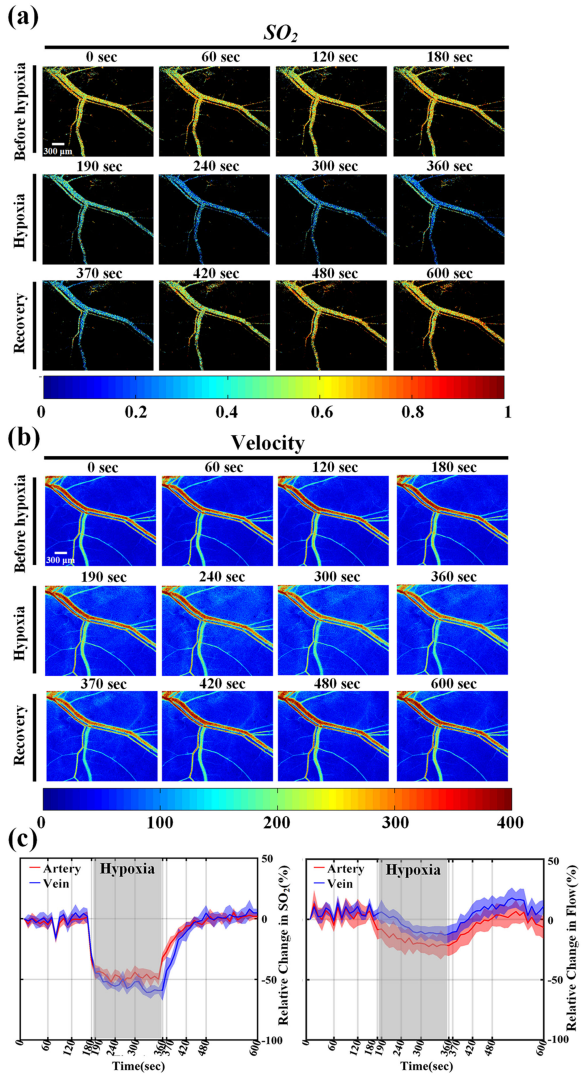

Fig. 4. Monitoring SO_2 and blood flow velocity changes in cutaneous microvessels. (a) The pseudo-colored images show the spatiotemporal changes of SO_2 maps in the cutaneous microvessels at initial, hypoxia and recovery phases through optical clearing skin window. (b) The pseudo-colored images show the spatiotemporal changes of blood flow velocity maps in the cutaneous microvessels at initial, hypoxia and recovery phases. (c) The statistic graphs show relative changes in SO_2 and blood flow velocity of the arteries and veins at different states. The gray shade area indicates the hypoxic stimulus phase. And the curves and the width of shadows are mean and standard deviation, respectively. (Scale bar: 300 μm).

D. The Comparative Analysis for Cerebral and Cutaneous Vascular Responses to Hypoxia

Meanwhile, we quantitatively compared the cortical and cutaneous vascular responses to the hypoxia. The hypoxic response rate was defined as the relative change rate between the moment of starting hypoxia and 20 sec after starting hypoxia, and the recovery rate was defined as the relative change rate between the moment of starting recovery and 20 sec after starting recovery. Fig. 5 shows that the hypoxic response rate and recovery rate of blood flow velocity in cerebral vessels are significantly higher than cutaneous vessels.

And as for hypoxic response rate of SO_2 in cerebral and cutaneous vessels, there was no significant difference for arteries but small significant difference for veins, and the recovery rate of SO_2 in cerebral was significantly higher than cutaneous vessels. These results indicated that cerebral vascular response was more sensitive than peripheral cutaneous vascular response to hypoxia, and the small decline of blood flow accompanied sharp decrease in SO_2 during hypoxia, but SO_2 did not continuously reduce but keep relatively stable.

IV. Discussion

At present, *in vitro* tissue optical clearing technique plays an important role in 3D visualization of tissues, which can significantly enhance the imaging depth and resolution, allowing to acquire complete structural information of tissue blocks [33],

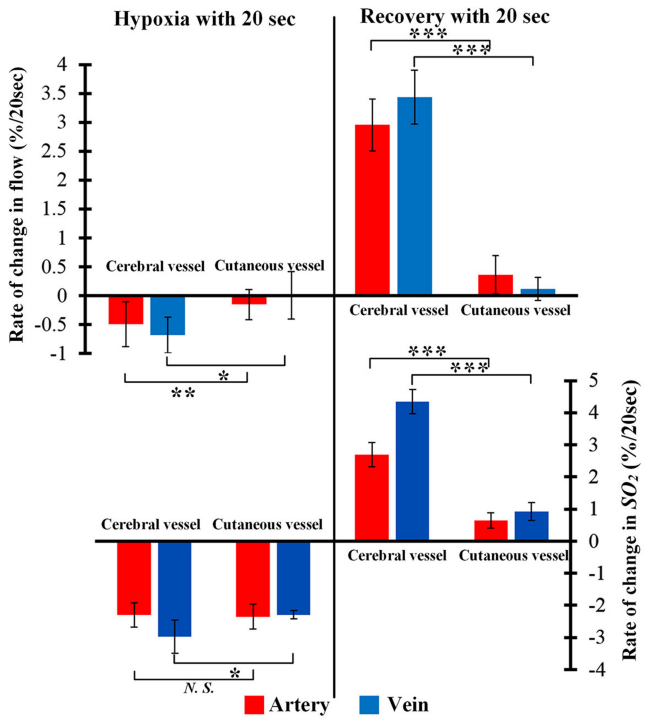

Fig. 5. The hypoxia rate and recovery rate of SO_2 and blood flow velocity in cerebral and cutaneous microvessels to hypoxia stimulus. The error bars indicate standard errors (S.E.). Data are shown as mean ± S.E., n = 5. $^{*}<0.05$, $^{**}<0.01$, $^{***}<0.001$, N.S. no significant difference.

[34]. However, for *in vivo* optical imaging, the turbid property of tissue, e.g., skull and skin, still severely limits the imaging performances. Therefore, various optical imaging windows based on the surgical operations have been developed, including many types of skin window models [9], [35] and cranial window models [36], [37]. Nevertheless, the surgical operation will inevitably cause a series of inflammatory reactions, which may affect the structure and function of vessels and cells. Here, the skin-OCA and skull-OCA were applied to dramatically improve the imaging qualities of HSI and LSCI, which have been verified to be safe, and they would not cause observable affects to the structure and function of cutaneous and cerebral vessels [38], [39]. By combining these two optical clearing windows with HSI&LSCI, we realized visualization of monitoring the changes in cortical and cutaneous arteriovenous blood oxygen and blood flow.

By now, many *in vivo* skin optical clearing methods have been developed and applied towards different skin sites, such as dorsal skin, ear skin and footpad skin [40]–[42]. In this study, the newly developed skin-OCA was employed, with the advantages of fast transparency and better effects. However, this method is more suitable for mice because their skin is much thinner than others, including pig, human, etc. Certainly, some other *in vivo* skull optical clearing methods also have been proposed, for instance, the skull-OCA consisted a mixture of liquid paraffin and Vaseline [43] as well as laurinol, weak alkaline substances, EDTA, dimethyl sulfoxide, sorbitol, and glucose [23], [44]. Although these methods have identified their effectiveness for improving imaging qualities, their repeatability and safety have not been verified. The *in vivo* skull clearing methods applied in this work has been identified to be safe, moreover, it allows to repeatedly applied to trace the cerebrovascular changes at the same region for 6 months [22]. However, it is unable to establish a permanent window by using this skull-OCA once. In the future, we will further optimize this skull optical clearing method to overcome this problem.

In this work, the cerebral and cutaneous vascular hemodynamic responses induced by hypoxia were monitored and quantitatively analyzed. Our results showed that the trend of blood oxygen response to hypoxia was more similar than that of blood flow response. It indicated that the changes in cortical and cutaneous vascular blood oxygen response to hypoxia might be more consistent than the blood flow. Although the trend of SO_2 in cutaneous vessels was similar with that of cerebral vessels, for the blood flow, the hypoxia-induced descent rate of cutaneous vessels was much lower than that of cerebral microvessels (Fig. 5). Additionally, we noticed that with the recovery of the breathing oxygen concentration, the cerebral blood flow increased immediately (Fig. 5), and the SO_2 could recover to the normal state instantly for rapid relief from the symptoms of hypoxia [45]. For the cutaneous microvessels, the recovery rates of both SO_2 and blood flow were slower than those of cerebral microvessels, which might attribute to the oxygen supply priority for the brain [45], [46]. In our recent work, we monitored the dynamic changes in the functional responses of cerebral and cutaneous vessels with the development of diabetes, and found that the cutaneous microvascular blood oxygen response had the potential to be an early warning target for the cerebrovascular dysfunction [30]. Additionally, some researchers suggest that there could be a link between cutaneous or systemic manifestations of amyloid deposits in Alzheimer's disease [47]. In fact, the skin offers an ideal detective site for investigating the peripheral and systemic responses to stress [48], and a stress-associated 'brain-skin connection' has been proposed [49]. By using some common and cost-effective detection methods on the skin, we can infer the dynamic changes of the cortex in some cases, avoiding the high cost or invasiveness for brain detection. Thus, the connections between brain and skin in various diseases is necessary to be investigated.

In hypoxia studies, isoflurane is often used for anesthesia to avoid the influence of body movements on imaging [50]. Some researchers made the comparation of hemodynamic changes in response to hypoxia between awake and anesthetized rat [51], and found that isoflurane could attenuate autonomic responses to hypoxia. This may be because isoflurane is a vasodilator, or isoflurane could abolish autoregulation of cerebral blood flow [52]. In this work, referring to the previous anesthetic strategies, the same concentration of isoflurane was kept during the whole experimental process to ensure the mice maintained at a relatively stable anesthetic state [50], [51]. And before the hypoxia stimulus, the SO_2 and blood flow were monitored for 180 seconds that fluctuated slightly as shown in Fig. 3(c) and Fig. 4(c). In the future, we will further improve and optimize the experimental techniques to realize the hemodynamic monitoring on awake mice, by which we can obtain more accurate vascular response excluding the effect of anesthesia. Similar

with other study, the cerebral blood oxygen decreased during the hypoxia [53]. Besides, our results showed that the cortical arteriovenous blood flow also decreased after hypoxia, which was consistent with René Schiffner *et al.*'s findings [54]. Ashley D. Harris' research found that across the grey matter, cerebral blood flow increased by approximately 15% over the course of hypoxia [55]. But the latest study reported that the subcortical blood flow increased, whereas the cortical blood flow decreased in comparison to baseline values during hypoxia [54]. These findings suggest that there may be differences in cerebral blood flow response in different brain regions to hypoxia.

The cerebral blood flow can be adjusted by systematic autoregulation, which depends on the duration of hypoxia, species, tissue type, as well as level and type of anesthetic [51], [56]. Additionally, a previous study has shown the general mathematical model between changes in cerebrovascular blood flow and oxygen metabolism, in which the blood flow and oxygen consumption are tightly coupled in a non-linear fashion [57]. As for the skin, it has been verified that there exists a causal relationship between skin blood flow and blood oxygen saturation [58]. And F. Tayyari *et al.* found that there was a relationship between retinal blood flow and blood oxygen saturation, but the correlations of the blood flow and blood oxygen saturation between veins and arteries were different [59]. In this study, we monitored the changes of blood flow and blood oxygen in cerebral and cutaneous arteriovenous microvessels during hypoxia, respectively. Our results are consistent with these previous studies. Besides, it demonstrates that the coupling between blood flow and oxygen metabolism in different tissues and organs may be different.

V. Conclusion

In this work, *in vivo* skull and skin optical clearing techniques were used for visualization of the cortical and cutaneous microvessels. And we combined the HSI and LSCI imaging systems to simultaneously monitor the cortical and cutaneous arteriovenous blood oxygen and blood flow changes to the hypoxic stimulus with the high spatial-temporal resolutions. The results demonstrated that the cutaneous microvascular blood oxygen response was similar with that of cortical microvessels under the hypoxic stimulus, but the trends of blood flow for cortical and cutaneous microvessels were different. Additionally, the quantitative results suggested that the cerebrovascular response was more sensitive than peripheral skin response to the hypoxic stimulus and recovery. This work provides a feasible approach to *in vivo* monitor cerebral and cutaneous microvascular functional responses, contributing to revealing the relations between cerebrovascular and peripheral circulation.

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