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Real-Time Visualization of Dextran Extravasation in Intermittent Hypoxia Mice using Non-Invasive SWIR Imaging

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Running head: SWIR imaging to monitor real-time extravasation *in vivo*

Key words: SWIR imaging, dextran extravasation, vessel permeability, intermittent hypoxia

New and noteworthy

We demonstrate that SWIR imaging technique is a useful tool to monitor real time dextran extravasation from vessels in vivo, with a high resolution. We report for the first time an increased real-time dextran (70kD) extravasation in mice exposed to intermittent hypoxia for 14 days compared to normoxic controls.

Abstract

Context. Imaging tools are crucial for studying the vascular network and its barrier function in various physiopathological conditions. Shortwave infrared window (SWIR) optical imaging allows non-invasive, in-depth exploration. We applied SWIR imaging, combined with vessel segmentation and deep learning analyses, to study real-time dextran probe extravasation in mice experiencing intermittent hypoxia—a characteristic of obstructive sleep apnea associated with potential cardiovascular alterations due to early vascular permeability. Evidence for permeability in this context is limited, making our investigation significant.

Methods. C57Bl/6 mice were exposed to normoxia or intermittent hypoxia for 14 days. Then SWIR imaging between 1250 and 1700 nm was performed on the saphenous artery and vein and on the surrounding tissue after intravenous injection of labeled dextrans of two different sizes (10 or 70 kDa). Post-processing and segmentation of the SWIR images were conducted using deep learning treatment.

Results. We monitored high-resolution signals, distinguishing arteries, veins, and surrounding tissues. In the saphenous artery and vein, post 70kD-dextran injection, tissue/vessel ratio was higher after intermittent hypoxia (IH) than normoxia (N) over 500 seconds ($p < 0.05$). However, the ratio was similar in N and IH post 10kD-dextran injection.

Conclusion. The SWIR imaging technique allows non-invasive real-time monitoring of dextran extravasation *in vivo*. Dextran-70 extravasation is increased after exposure to IH, suggesting an increased vessel permeability in this mice model of obstructive sleep apnea.

Introduction

Monitoring vascularization in vivo at high spatial and temporal resolutions is considered a crucial aspect for understanding the onset of severe pathologies, including cardiovascular diseases and oncology (1, 2).

While Magnetic Resonance Imaging and Positron Emission Imaging are standard techniques for visualizing the vascular network in depth, optical imaging offers distinct advantages due to its high spatial and temporal resolution, relatively affordable and miniaturized equipment, facilitating rapid translation to clinics and hospitals. For example, real-time optical vascular imaging using wide-field imaging or optical coherence tomography has been employed in hospitals to assess various pathologies, including the microvascular circulation of myofascial free flaps in the head and neck region.(3) or to monitor retinal ocular diseases(4).

One of the challenge of optical imaging for clinical translation is related to the difficulty to reach high depth due to the absorption, the autofluorescence and the scattering of living tissues(5, 6). A strategy to overcome this obstacle consists in detecting at higher wavelength in the shortwave infrared window (SWIR) between 1000 and 2100 nm called also the NIR-II region. Hongjie Dai's team was pioneer in this field and demonstrated in 2012 and 2014 the benefit moving from the NIR-I (700-900 nm) to the SWIR region reaching high resolution up to 10 microns down to few millimeters depth(7, 8) . The main reason is associated to the exponential decrease of light scattering at higher wavelengths in the living tissue combined with the very low autofluorescence(6). Since then, extensive efforts have been devoted to improving the sensitivity of sensors in the SWIR by developing new fluorophores within this spectral window (9–13). These efforts aim to assess the potential of SWIR imaging for various biomedical applications, including cardio and cerebrovascular diseases(14, 15), oncology(16–18), neurodegenerative pathologies(19), among others (20, 21). A significant advance in the clinical translation of new SWIR probes was reported by Bawendi's team, demonstrating the capability to track FDA-approved organic dyes, Indocyanine Green (ICG) and IRDye800Cw, through in vivo SWIR imaging(22).

In parallel, we are witnessing the rapid development of image post-processing, notably with the use of Monte Carlo restoration and artificial intelligence segmentation for SWIR bioimaging(23–25). This allows for higher contrast and spatial resolution in SWIR images, providing real-time segmentation and analyses. In some cases, it even enables flow mapping of the vascular network(23) or non-invasive confirmation of vascular disorders in mice (25). The application of deep learning in image analysis can also automate the real-time segmentation of the vascular network, opening up future possibilities for optical-guided surgery and the development of the next generation of point-of-care

devices. The segmentation and digitalization of vessels also represent a significant step toward digital life systems(26).

Leveraging our capacity to investigate vascular networks in real time and with high resolution, we propose that the utilization of SWIR imaging could serve as a viable method to monitor the extravasation of fluorescent biomarkers from rodent vasculature in different physio-pathological contexts.

Intermittent hypoxia (IH) is one of the major hallmarks of obstructive sleep apnea (OSA), a respiratory disease characterized by recurrent airway obstruction during sleep, leading to repeated desaturation-reoxygenation cycles, known as intermittent hypoxia(27). IH is strongly associated with cardiovascular alterations(27) such as increased blood pressure(28) and atherosclerosis(29, 30), resulting in an increased risk of myocardial infarction(31) and stroke(32). Vascular dysfunction, including dysregulation of vascular permeability, could be one of the early steps leading to atherogenesis. Recent data suggest that IH could be associated with increased vascular permeability, although evidence is still scarce and mainly arising from in vitro studies(33–36). Permeability was studied by assessing trans-endothelial electrical resistance (TEER) or fluorescent molecules (FITC-dextran or NaFl) transendothelial passage, in IH models using endothelial cell models (Human lung endothelial cells(34)), mouse microvascular bEnd.3 cells(33) or human aortic endothelial cells(36)), or using a blood-brain barrier model including bEnd.3 cells and C6 astrocytes(35). Authors consistently found a 20-50% decrease of TEER or increase of permeability in these models. Among the possible mechanisms leading to increased vascular permeability in OSA, we recently demonstrated that IH can cause the cleavage of VE-cadherin, a major protein of adherent junctions between endothelial cells and a crucial regulator of endothelial permeability(29, 36). However, increased vascular permeability has not been demonstrated *in vivo* so far.

Thus, our objective was to use SWIR imaging and vessel segmentation methods based on deep learning to investigate vascular permeability through dextran extravasation in mice exposed to 14 nights of IH.

Materials and methods

Dextran labeling:

IRdye800Cw NHS ester (Mw= 1165.20 $g.mol^{-1}$) was purchased from Li-Cor and the amino dextran 10k Da and 70kDa were obtained from Invitrogen. We used a standard labeling to couple the dye to the dextran(37).

Briefly, IRdye800Cw NHS ester was dissolved at 10 mg/mL in DMSO (8.6 mM) and Amino-dextran 10,000 and 70,000 were dispersed at 10 mg/mL in PBS buffer (10 mM) pH 8. We added 1 to 4 times the dyes in excess with respected to the dextran sols and stir 90 min which correspond in theory to 0.5 to 2 dyes per dextran for 10 kDa and to 3 to 6 dyes per dextran for 70 kDa. We then purify the labeled Dextran 10kDa (D-10) and Dextran 70kDa (D-70) with sephadex-25 column 5 times to remove the free dyes. Absorbance spectra of the labeled dextran 10kDa and 70kDa were measured to determine the degree of IRdye800Cw labeling of the two types of Dextran.

Animals

We used 16 wild type C57BL/6J mice (males, age 12 weeks, weight 26-27 g) purchased from Janvier Labs (Le Genest-Saint-Isle, France). All mice were fed on a standard diet and were weighed every week of exposure to IH. Animals were housed in a local facility (at 20-22°C) and had free access to food and water. The study was conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986), and to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Our project was approved by the institutional ethical committee (University Grenoble Alpes, Cometh n°12) and the French Ministry of Research (APAFIS#22190-2019072615145639).

Exposure to intermittent hypoxia

Wild type (WT) C57BL/6J mice were randomly exposed to IH or N for 2 weeks as described before(29). The fraction of inspired oxygen (FiO_2) in cages was monitored with a gas analyzer throughout the experiments. Intermittent hypoxia was generated by intermittent injection of low oxygen air in the cages, with 1-minute cycles (30 seconds at 5% FiO_2, 30 seconds at 21% FiO_2 in the cages) repeated for 8 hours/day (from 8:00am to 4:00pm). Normoxic mice were exposed to the same air flow turbulences and noises as IH mice.

In vivo SWIR imaging

Dextran SWIR imaging was performed after 2 weeks of IH or normoxia exposure. The day before imaging, mice were anesthetized (air/isoflurane 4% for induction and 1.5% thereafter) and depilated on the thigh zone. On the injection day, mice were anesthetized with isoflurane, a catheter was

introduced in the tail vein and fluorescent-labelled Dextrans of 2 sizes were injected: D10-IR800 or D70-IR800). For each mouse, SWIR imaging was performed twice, first in the morning using Dextran10-IR800 (200 µL at 5 mg/mL) because of his fast clearance, and then in the afternoon using Dextran70-IR800 (200 µL at 5 mg/mL). The complete elimination of the D10 signal was checked before the administration of the D70.

Real-time image acquisition started a few seconds before Dextran injection via the catheter and image capture was focused on the vessels of the tight and in particular on the saphenous artery and vein which were analyzed (supporting movie M1). Image acquisition and analysis was performed blindly, with manipulators not knowing if the mice were exposed to N or IH.

SWIR imaging was performed using a Princeton camera 640ST (900-1700 nm) coupled with a laser excitation source at λ= 808 nm (100 mW/cm^2). We used short-pass excitation filter at 1000 nm (Thorlabs) and long pass filters on the NIR-II camera at 1250 nm (Thorlabs). A 50 mm lens with 1.4 aperture (Navitar) was used to focus on the region of interest. All images were captured continuously for 500s with a 1s exposure time for the temporal analysis of vascular dynamics.

Image post-processing

All acquired images underwent the same preprocessing steps to enhance image quality using FIJI software. This included flat field correction, dark current subtraction, and background normalization. The acquired SWIR images were acquired in grey color space, where grey colors correspond to intensity values, which were analyzed in the next step.

Vessel Segmentation

We employed the IterNet neural network(38) to forecast vessel patterns within SWIR images, utilizing the universally pre-trained weights provided by the authors. This approach has demonstrated exceptional segmentation performance specifically tailored for SWIR image analysis(23) Based on the identified maximum intensity values, masks are created to segment the arteries, veins, and tissue from the background. The masks are generated by thresholding the intensity values in the original SWIR images. Pixels with intensity values falling within the threshold range for each vessel type are considered part of the vessel mask. The vessels (saphenous vein and artery) have elongated masks along the vessel, while the tissue mask is a square in a characteristic zone of the tissue with no visible vessels.

Histogram Analysis

This technique is based on histogram analysis of the grey color intensity values across the vessels and tissue selected by the corresponding masks. The histogram analysis is performed to identify the signal intensity values per pixel associated with arteries, veins, and tissue, correspondingly. This analysis is carried out on each frame of the acquired SWIR images. The following steps are performed for each frame:

a. Calculation of Intensity Histogram:

A histogram of the grey color intensity values is computed within the masks in a current frame. The intensity values ranging from 0 (black) to 255 (white), and the histogram binning equal to 100.

b. Intensity Plots

The average intensity values in the masked window, obtained from the histogram analysis for saphenous artery and vein, and adjacent tissue are plotted as a function of time (supporting movie M1). These intensity plots provide insights into the temporal changes in the vascular structures and can be further analyzed to extract relevant physiological information.

c. Tissue/vessel ratio calculation

Ratios of tissue to artery signals (T/A) and tissue to vein signals (T/V) were calculated along time to report for Dextran tracer diffusion from the corresponding vessel toward the adjacent tissue.

Statistics

Due to mice death or problems during data acquisition, the final number of mice is n=6 in the normoxic group and n= 5 in the hypoxic group. Indeed, two mice died during the acquisition process (one in the IH group and one in the normoxic group). Their death was attributed to anesthesia management trouble and not to the SWIR technique itself. On the other hand, some imaging data could not be exploited because image quality was not good enough (insufficiently sharp focus setting) for efficient automatic segmentation of the vessels all along the kinetic. When only one of the two acquisition sets (D10 or D70) was of insufficient quality, all data from this mouse had to be excluded from the study.

For each type of dextran (D10/D70), ratios of tissue to artery fluorescence signals (T/A) and Tissue/Vein fluorescence signals (T/V) were calculated over the acquisition kinetics of each mouse and two-way ANOVA analyses were performed using GraphPad Prism version 10 (GraphPad software, USA) to compare the kinetics of the N and IH groups.

Results and discussion

SWIR in vivo optical imaging conducted in our platform allows us to achieve spatial resolution in depth down to a few millimeters (~4 mm)(25) at the micrometric scale. This enables the confident distinction between vessels and arteries. Additionally, non-invasive measurements have been performed at a rate of 500 ms per frame, providing sufficient temporal resolution to monitor the kinetic behavior of dextran—from arteries and veins to its diffusion in the adjacent tissue—by selecting the leg of the mice in both normoxia (N) and intermittent hypoxia (IH) models. Representative images and movie are provided in supplementary material (Figures S1, S2, Movie S3). Image processing through deep learning as shown in Figure 2 enhances the contrast and spatial resolution for each SWIR image, allowing for the segmentation of the vascular network.

In the saphenous artery, the tissue/artery signal ratio was similar between N and IH mice after the injection of a 10kD fluorescent dextran (Figure 3A). Conversely, after injecting the 70kD dextran, the tissue/artery signal ratio was significantly higher in IH mice than in N mice ($p=0.022$) over 500 seconds post-injection (Figure 3B). Similarly, for the saphenous vein, there was no difference in the tissue/vein signal ratio after the injection of 10kD dextran (Figure 3C). However, after injecting the 70kD dextran, the tissue/vein signal ratio was significantly higher in IH mice than in N mice ($p=0.025$) (Figure 3D).

Given that blood flow could influence the kinetics of signal detection in vessels and tissues, we normalized the tissue signal by calculating the tissue/vessel signal ratio to mitigate potential blood flow effects.

Our results suggest increased vessel permeability leading to enhanced extravasation of dextran towards surrounding tissues, both from arteries and veins. To our knowledge, this is the first time that both artery and vein permeability have been studied in vivo and in real time in mice exposed to intermittent hypoxia. Previous findings suggested cerebrovascular permeability after IH or sleep fragmentation *in vitro* (33, 35) and *in vivo* (39, 40) although conflicting results also reported no impact of IH on the blood-brain barrier permeability(41, 42). On systemic vessels, increased vessel permeability was reported after IH in vitro, mainly on arterial or microvascular cells (34, 36), but in vivo data are scarce, with only one paper investigating aortic permeability(33). These studies typically rely on dextran or Evans Blue injection with an end-point measurement on a whole tissue lysate. In contrast, the strength of our method lies in the real-time assessment of dextran extravasation, for various time periods, from specific arteries or veins to adjacent tissue. While we may hypothesize that similar findings could be found in other systemic vessels with similar diameter and physiological

properties, further studies should investigate larger or smaller vessels. In particular, microcirculation in ears or paws could be easily accessible using this imaging technique.

An interesting future aspect to explore would be to investigate the kinetics of diffusion from arteries/veins to the tissue as a function of fluorophore size, testing labeled dextran in the range between 10 and 70 kDa. This may improve accuracy in determining dextran permeability of vessels and correlate with the severity of hypoxia in mice. Another perspective could be to investigate variations of permeability in different IH models, with different IH duration or severity, or in old/young or male/female mice.

The combination of SWIR imaging collected above 1250 nm and the improved contrast and spatial resolution by deep learning allows for very detailed segmentation of the vascular network down to a few millimeters. The segmentation method(23) relies on the inherent structural redundancy or self-similarity within blood vessels, enabling the network to discern obscured details of the vessel from the segmented image.

In conclusion, our study describes a novel, sensitive method for real-time in vivo imaging of dextran extravasation and demonstrates, for the first time, increased permeability of the saphenous artery and vein after exposure to IH. This strengthens the current understanding of the mechanisms leading to cardiovascular alterations in obstructive sleep apnea (OSA). Moreover, it demonstrates the feasibility of non-invasive imaging follow-up over time (i.e., disease progression or aging), opening new avenues for investigating vascular physiology in various physiological or physio-pathological contexts, including detection and monitoring of sub-surface vascularization and permeability in different types of pathologies such as Raynaud's syndrome, sclerodermia or microvascular complications of diabetes mellitus, at the condition that suitable contrast agents should first be approved for human use.

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Disclosures

The authors declare that they have no conflict of interest.

Supplementary material

Supplementary figures S1, S2 and movie S3 can be found online at https://osf.io/523nq/?view_only=80218c3b93604ddaae0cae68d2b347ae

Author contributions

XLG, VJ and ABM conceived the study; OH and MH performed experiments; VB analyzed images; XLG, VJ and ABM analyzed data and drafted the manuscript. All authors approved the final version of manuscript.

Figure legends

Figure 1 : Protocol to expose mice to intermittent hypoxia in mice model and SWIR imaging platform using near infrared laser excitation ($\lambda_{exc.}$ 808 nm at 100 mW/cm^2; $\lambda_{em.}$ [1250-1700 nm].

Figure 2 : Image post-processing, segmentation and analyses assisted by deep learning on N and IH mice.

Figure 3 : Intermittent hypoxia increases Dextran 70 extravasation from saphenous artery and vein.

Tissue to vessel fluorescence signal ratios were calculated to report Dextran extravasation from saphenous artery (A and B) or vein (C and D), for Dextran10-IR800 (A and C) or dextran70-IR800 (B and D). n= 5-7 mice/group. Statistical analysis: Two-way ANOVA, * $p<0.05$.