

Photodynamic opening of the blood-brain barrier affects meningeal lymphatics and the brain's drainage in healthy male mice

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Abstract: Here, we present the new vascular effects of photodynamic therapy (PDT) with 5-aminolevulinic acid (5-ALA). PDT with 5-ALA induces a leakage of both the meningeal and cerebral blood vessels. The extravasation of photo-excited 5-ALA from the leaky blood vessels into the meninges causes photo-damage of the meningeal lymphatics (MLVs) leading to a dramatic reducing the MLV network and brain's drainage. The PDT-induced impairment of lymphatic regulation of brain's drainage can lead to excessive accumulation of fluids in brain tissues, which is important to consider in the PDT therapy for brain diseases as a possible side effect of PDT with 5-ALA.

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1. Introduction

Photodynamic therapy (PDT) is a promising add-on method to the current standard of care for patients with brain tumors [1–7]. This method of therapy consists of introducing into the blood or locally a photosensitizer (PS), which specifically accumulates in tumor cells and, due to photoexcitation, singlet oxygen is formed, causing the death of tumor cells. The 5-aminolevulinic acid (5-ALA) is PS, which was approved by the United States Food and Drug Administration for PDT of glioblastoma [5–7]. A detailed study of the mechanisms of action of 5-ALA on the cerebral vessels revealed that in addition to its suppressive effect on tumor cells, 5-ALA induces opening of the blood-brain barrier (OBBB) [8–14]. Indeed, 5-ALA causes a reversible increase in the BBB permeability to low and high molecular weight compounds in a dose-related manner [9]. Studies in mice have shown that other PSs can also cause OBBB [14].

Recently, it has been shown that PDT with an injection of PS into the cisterna magna (i.c.m.) induces photoablation of the meningeal lymphatics (MLVs) playing an important role in brain's drainage and lymphatic removal wastes, metabolites and toxins from the brain [15–19]. So, Da Mesquita et al. clearly show damage of MLVs by an injection of visudyne into the cisterna magna (i.c.m.) and its photoexcitation by a laser with wavelength 689 nm (50 J per cm² at an intensity of 600 mW per cm² during 83 s) [16,17]. PDT was performed in the sides of main venous sinuses, including the superior sagittal sinus (SSS) and the transverse sinuses (TS). Visudyne diffuses into the cerebral spinal fluid (CSF) and during photoexcitation, singlet oxygen is formed, which

damages MLVs located along the venous sinuses. The photoablation of MLVs is associated with a dramatic suppression of brains' drainage and lymphatic removal of amyloid beta ($A\beta$) from brain tissues [16]. Later, these authors reported that this method affects only dorsal MLVs along the SSS and TS, i.e. in the place of laser irradiation and does not influence on the basal MLVs around the sigmoid sinus and the petrosquamosal sinus, which are located in deeper areas of the brain and where laser is not applied [17]. However, even when part of MLV network remains intact in MLV-defective mice, the excretion of $A\beta$ into the deep cervical lymph nodes (dcLNs) is significantly reduced. Hu et al. using the magnetic resonance imaging in mice with brain tumor found that photo-ablation of dorsal MLVs aggravated cerebral edema suggesting PDT-mediated reducing intratumor drainage [18]. Li et al. show in the experiments on mice with a model of intraventricular hemorrhage that MLVs play a critical role in removal of red blood cells from the brain to dcLNs that is significantly suppressed after PDT with visudyne [19]. In our previous study, we modified the method of PDT-injury of MLVs using 5-ALA as PS instead of visudyne [20]. Our results clearly demonstrate that PDT with 5-ALA (i.c.m.) effectively damages MLVs and reduces brain's drainage that are associated with a decrease in lymphatic removal of $A\beta$ from the brain to the peripheral lymphatics. Thus, photoexcitation of 5-ALA affects the meningeal network contributing a lymphatic dysfunction.

Since 5-ALA, when administered intravenously, can cause OBBB [8–14], we hypothesized that it also can increase the permeability of meningeal blood vessels that may lead to damage to MLVs and brain's drainage. Testing this hypothesis is important to better understand the mechanisms of the vascular effects of PDT and optimize the current guidelines for PDT using 5-ALA.

Here, we studied the PDT effects with 5-ALA (i.v. or i.c.m.) on both the meningeal and cerebral blood vessels. In the first step, we analyzed lymphatic removal of contrast agent from the brain to dcLNs in sham mice and in PDT-induced MLV-defected animals. In the second step, we evaluated the PDT effects with 5-ALA (i.v. or i.c.m.) on the dorsal MLV network covering the main venous sinuses (SSS and TS) using confocal analysis of the MLV density and the specific marker of the lymphatic endothelium. In the third step, we investigated the PDT-mediated increase in the permeability of meningeal and cerebral blood vessels using real time multiphoton microscopy through the optical window to maintain integrity of MLVs as well as ex vivo confocal imaging.

2. Methods

2.1. Subjects

Male adult C57BL/6 mice (23–25 g, 3 months age) were used in all experiments. Mice were purchased from the National Laboratory Animal Resource Centre in Pushchino (Moscow area, Russia). The animals were housed under standard laboratory conditions ($25 \pm 2^\circ\text{C}$, 55% humidity, and 12:12 h light–dark cycle) with access to food and water ad libitum. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes, and the guidelines from the Ministry of Science and High Education of the Russian Federation (№742 from 13.11.1984), which have been approved by the Bioethics Commission of the Saratov State University (Protocol No. 8, 18.04.2023). The experiments were performed in the following groups: (1) the control (intact mice); (2) 5-ALA i.v. without laser; (3) 5-ALA i.c.m. without laser; (4) laser without 5-ALA; (5) PDT with 5-ALA i.c.m.; (6) PDT with 5-ALA i.v.; $n = 8$ in each group in all sessions of the experiments.

2.2. *PDT-induced OBBB*

The non-invasive method of photoexcitation of 5-ALA injected into the blood, was used to OBBB as previously described [13]. Briefly, mice were anaesthetized by with 1% isoflurane (Sigma-Aldrich, St Luis, USA, at rate 1 L/min N₂O/O₂—70/30 ratio), and fixed in a stereotactic frame. 5-ALA (20 mg/kg, 5 μ L, Niopik, Moscow, Russia) was administered as a bolus intravenous injection. Thirty minutes later, a laser (XPeBRD-L1-0000-00901, CREE, Inc., Durham, North Carolina, USA), which emits a 1 W maximum light power at 635 nm with a light dose of 15 J/cm² was applied through a thinned skull [21].

2.3. *Assessment of OBBB*

To study the BBB leakage, we used three protocols based on intravenous injection of Evans Blue dye (2 mg/25 g mouse, 1% solution in physiological 0.9% saline, Sigma Chemical Co., St. Louis, MI, USA) [22,23]. For in vivo imaging of Evans Blue extravasation from the cerebral vessels into the brain parenchyma, we used multiphoton laser scanning microscope (Nikon A1R MP, Nikon Instruments Inc., Tokyo, Japan) and observed the permeability of microvasculature in the cortex (depth 150 μ m) during 30 min 1 h after PDT with 5-ALA (i.v.). For ex vivo imaging of the permeability of the meningeal and cerebral vessels, Evans Blue was allowed to circulate in the blood during 30 min. Afterward, the meninges and the brains were collected, and fixed in 4% paraformaldehyde for 12 hrs and 24 hrs, respectively. The whole meninges and the 50- μ m brain slices (they were cut on a vibratome Leica VT 1000S Microsystem, Germany) were analyzed on a confocal laser scanning microscope (Nikon A1R MP, Nikon Instruments Inc., Tokyo, Japan). For quantitative analysis of the Evans Blue level in the meninges and the brains, the tested tissues were collected and placed on ice with further preparation for the spectrofluorometric analysis according to the adapted protocol published by Wang et al. [23].

2.4. *PDT-induced damages of MLVs*

5-ALA (20 mg/kg, 5 μ L, Niopik, Moscow, Russia) was injected at a speed of 0.1 μ L/min into the cisterna magna under anesthesia with 1% isoflurane (Sigma-Aldrich, St Luis, USA, at rate 1 L/min N₂O/O₂—70/30 ratio) and was excited by the laser 635 nm (XPeBRD-L1-0000-00901, CREE, Inc., Durham, North Carolina, USA) with a light dose of 15 J/cm² [20]. The laser was applied through the intact skull in different regions, including the cisterna magna, SSS and TS. To quantify the MLV network covering SSS and TS, images were opened in the Fiji open-source image processing package [24]. The region of interest (ROI) manager was used for the analysis of total MLV length and branches in ROI as previously described [17]. The coverage by LYVE-1 + vessels was expressed as a percentage of ROI (% of ROI) at SSS and TS.

2.5. *Optical imaging of lymphatic clearance of tracer from the brain*

7 days after photo-ablation of MLVs, we studied brain drainage in mice with defected MLVs. With this aim, the fluorescein Isothiocyanate-Dextran (FITCD, Sigma-Aldrich, St Luis, USA) 70 kDa was injected into the right lateral ventricle (AP—0.5 mm; ML—1.2 mm; DV—2.0 mm at a rate of 0.1 μ L/min) through the chronical polyethylene catheter (PE-10, 0.28 mm ID $\times$ 0.61 mm OD, Scientific Commodities Inc., Lake Havasu City, Arizona, USA) using microinjector (Stoelting, St. Luis, USA) and allowed to spread in CSF during 1.5 hrs. Afterward, ex vivo imaging of the whole brain from bottom aspect as well as dcLNs was performed using Nikon A1R MP upright confocal microscope (Nikon Corp., Tokyo, Japan) with CFI Plan Apochromat Lambda D 2X (Nikon Corp., Tokyo, Japan) installed in a focusing nosepiece. Brain samples were submerged in a buffer solution in a Petri dish placed on the microscope stage. The top surface of each sample was covered with a 25mm $\times$ 25mm $\times$ 0.17 mm glass cover slide. Each brain image was captured as a stack of 5 stitched large images over 4 $\times$ 4 fields of view each. Image

resolution was 3636×3636 pixels at $6.11 \mu\text{m}/\text{pixel}$. Z step was $222 \mu\text{m}$. The resulting image was obtained as maximum intensity Z projection of all 5 images of the stack. The method enables to obtain extended along 1 mm depth of focus of high resolution image. The confocal images were captured in two channels: 488 nm excitation, 525/50 nm emission was used to image FITCD distribution; and 640 nm excitation, 700/75 nm emission for EB. dcLNs were imaged identically but only single field of view was captured for each stack. Image processing was performed using Fiji open-source image processing package [24]. Image processing procedures were identical for each pair of images (control and laser-treated samples) for each channel to ensure an accurate comparison of the fluorescence intensity.

2.6. Statistical analysis

All statistical analysis was performed using Microsoft Office Excel and SPSS 17.0 for Windows software. The results were reported as a mean value $\pm$ standard error of the mean (SEM). The inter-groups differences in all series experiments were evaluated using the ANOVA test with the post hoc Duncan test. The significance levels were set at $p < 0.05$ for all analyses.

3. Results

3.1. PDT effects on brain's drainage

In the first step, we studied brain's drainage using the ex vivo confocal analysis of the whole brain imaging and removal of FITCD from the brain to the peripheral lymphatic system. With this aim, 7 days after photoablation of MLVs by PDT, FITCD was injected into the right lateral ventricle and was distributed over the brain for 1.5 hrs, followed by release into dcLNs. There were no any changes in the fluorescent signal from FITCD in the brain and dcLNs between the control groups, including 5-ALA (i.c.m.) or 5-ALA (i.v.) administration without laser excitation as well as laser irradiation alone (Fig. 1(a)-(d)). However, PDT in both groups, including laser excitation of 5-ALA (i.c.m.) and 5-ALA (i.v.) caused a significant decrease in the fluorescent signal from FITCD in the brain and dcLNs (Fig. 1(a)-(d)). The quantitative analysis revealed that PDT-mediated reducing brain's drainage was stronger in the PDT group received 5-ALA (i.c.m.) compared with the PDT group treated by 5-ALA (i.v.). Thus, these data suggest that PDT induces suppression of brain's drainage leading to reducing lymphatic cleansing of brain tissue that is observed in both types of 5-ALA injection, into the blood circulatory system and especially into the cisterna magna.

3.2. PDT effects on the dorsal MLV network

Since in the first series of experiments we found a decrease in brain's drainage after PDT, at the next step we aimed to study of the PDT effects on the dorsal MLV network covering the main venous sinuses (SSS and TS), which plays a key role in brain's drainage [25] (Fig. 2(b)). Figure 2(a) clearly demonstrates that the coverage of LYVE-1 + vessels along SSS and TS was significantly reduced 7 days after PDT in mice treated by 5-ALA (i.c.m.) and 5-ALA (i.v.) compared with the control (intact mice). However, the quantitative analysis revealed that photo-damages of MLVs was less after PDT + 5-ALA (i.v.) vs. PDT + 5-ALA (i.c.m.) (Fig. 2(c)). These results suggest that reducing brain's drainage in the PDT groups can be related to dramatically decrease in the dorsal MLV network.

3.3. PDT effects on the permeability of the meningeal and cerebral vessels

To confirm the hypothesis that PDT causes the leakage of both the cerebral and meningeal blood vessels, we analyzed the permeability of the meningeal and cerebral vasculature to Evans blue before and 1 h after PDT with 5-ALA (i.v.) and 5-ALA (i.c.m.). Figure 2(a) shows extravasation of Evans Blue from the meningeal blood vessels into the CSF-filled space of the meninges in both

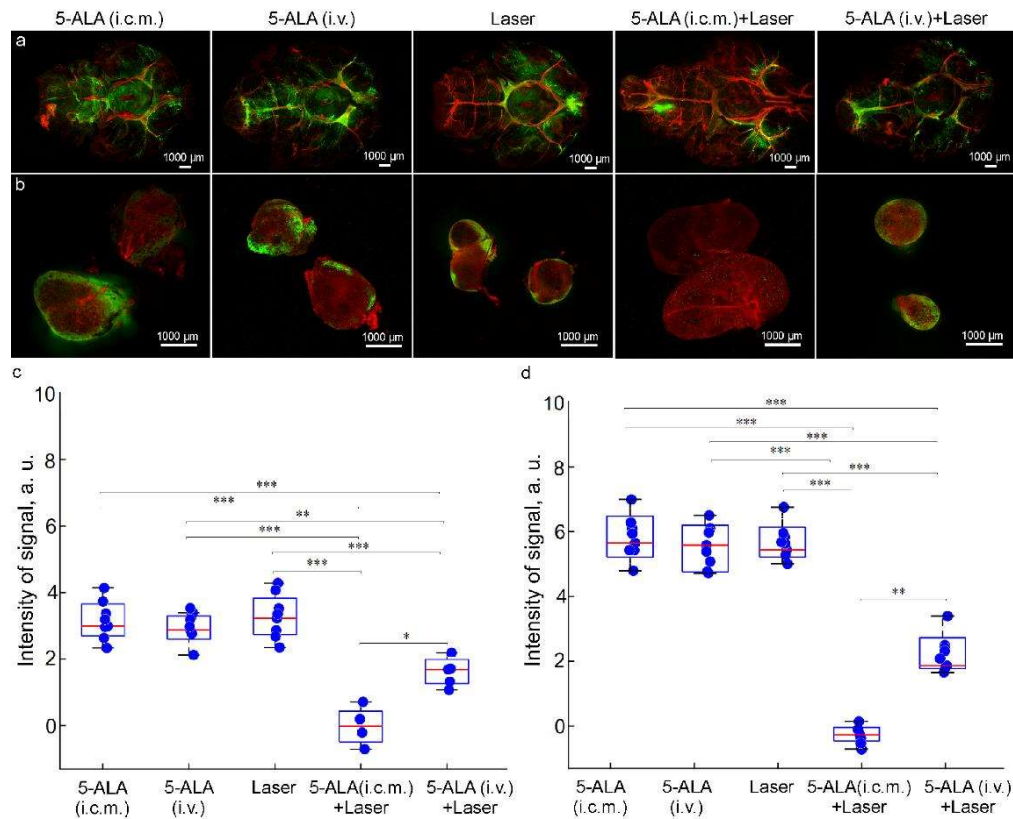


Fig. 1. The PDT effects on FITCD distribution in the brain and its accumulation in dCLNs: (a and b) Representative images of the bottom aspect of the brain (a) and dCLNs (b) before PDT in the control groups, including mice treated with 5-ALA (i.c.m.), 5-ALA (i.v.) or laser alone and 7 days after PDT in the 5-ALA (i.c.m.) + Laser and the 5-ALA (i.v.) + Laser groups; (c and d) Quantitative analysis of the signal intensity from FITCD in the brain (c) and in dCLNs (d) in the tested groups, $n = 8$ in each group, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, the ANOVA test with post hoc Duncan test.

the PDT groups with 5-ALA (i.v.) and 5-ALA (i.c.m.). Spectrofluorometric analysis revealed the high level of Evans Blue in the meninges after PDT vs. the control group ($0.69 \pm 0.03 \mu\text{g/g}$ tissue vs. $0.09 \pm 0.01 \mu\text{g/g}$ tissue, $p < 0.001$ between the PDT group with 5-ALA i.c.m. and the control, respectively; $0.54 \pm 0.02 \mu\text{g/g}$ tissue vs. $0.09 \pm 0.01 \mu\text{g/g}$ tissue between the PDT group with 5-ALA i.v. and the control, respectively; $n = 8$ in each group, the ANOVA test with the post hoc Duncan test).

However, only in the PDT group with 5-ALA i.v. but not in the PDT group with 5-ALA i.c.m. was observed OBBB in the cortex. Figures 3(b) and (d) illustrate the vivo multiphoton and ex vivo confocal imaging of the BBB leakage in mice after PDT with 5-ALA i.v., respectively. Figure 3(a) and (c) show no OBBB in the control group without PDT. Quantitative analysis demonstrates the high level of Evans Blue in brain tissues compared with the control group ($1.17 \pm 0.03 \mu\text{g/g}$ tissue vs. $0.12 \pm 0.02 \mu\text{g/g}$ tissue between the PDT group with 5-ALA i.v. and the control, $n = 8$ in each group, the ANOVA test with the post hoc Duncan test).

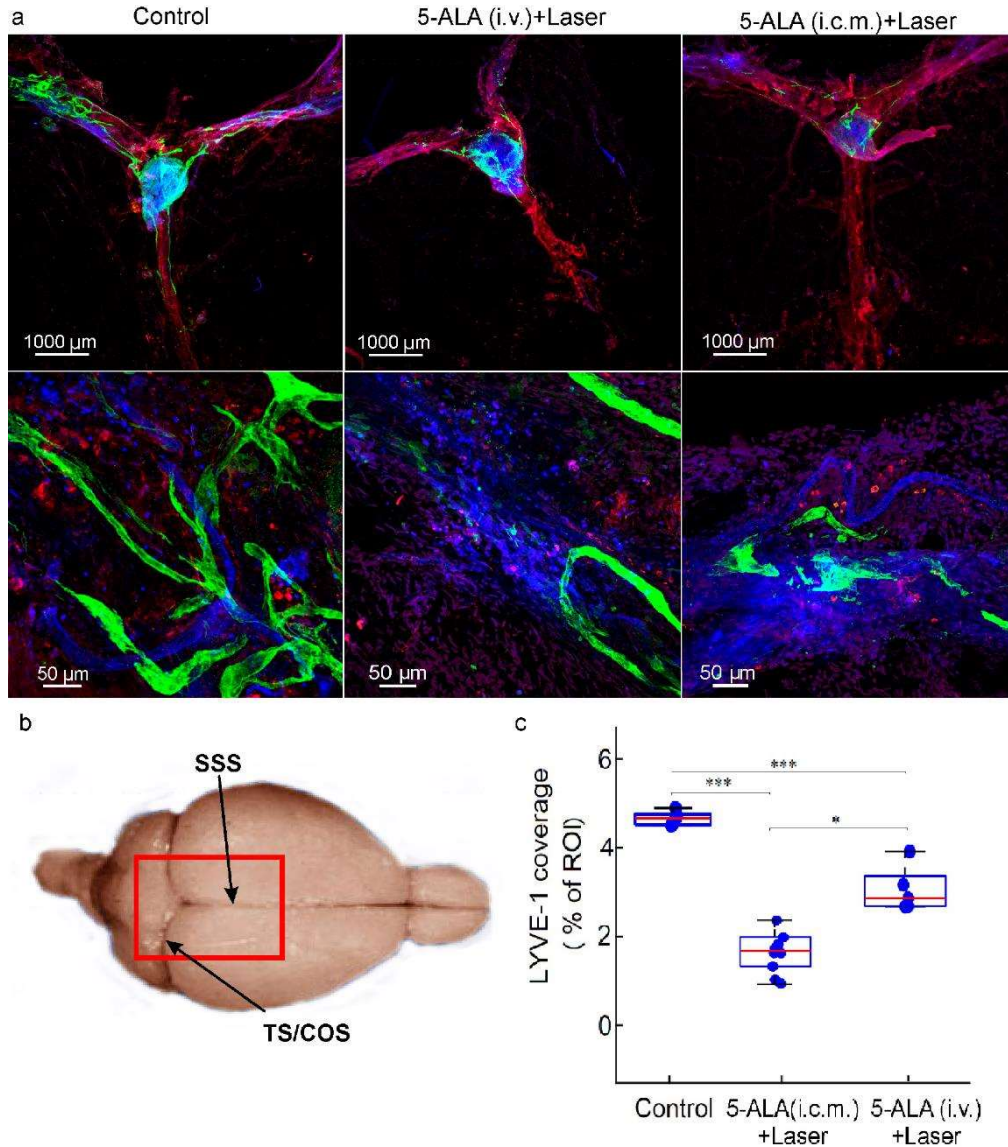


Fig. 2. The PDT effects on the dorsal MLV network and the permeability of the meningeal blood vessels: (a) Representative images of LYVE-1 + vessels (green) covering the main venous sinuses (SSS and TS) in the control and PDT groups. The blood vessels are marked with CD 31 (red) and filled with Evans Blue (blue), DAPI (violet); (b) the schematic illustration of ROI for z confocal analysis of the dorsal MLVs; (c) Quantitative analysis of LYVE-1 coverage expressed in % of ROI, $n = 8$ in each group, * - $p < 0.05$, *** - $p < 0.001$, the ANOVA test with post hoc Duncan test.

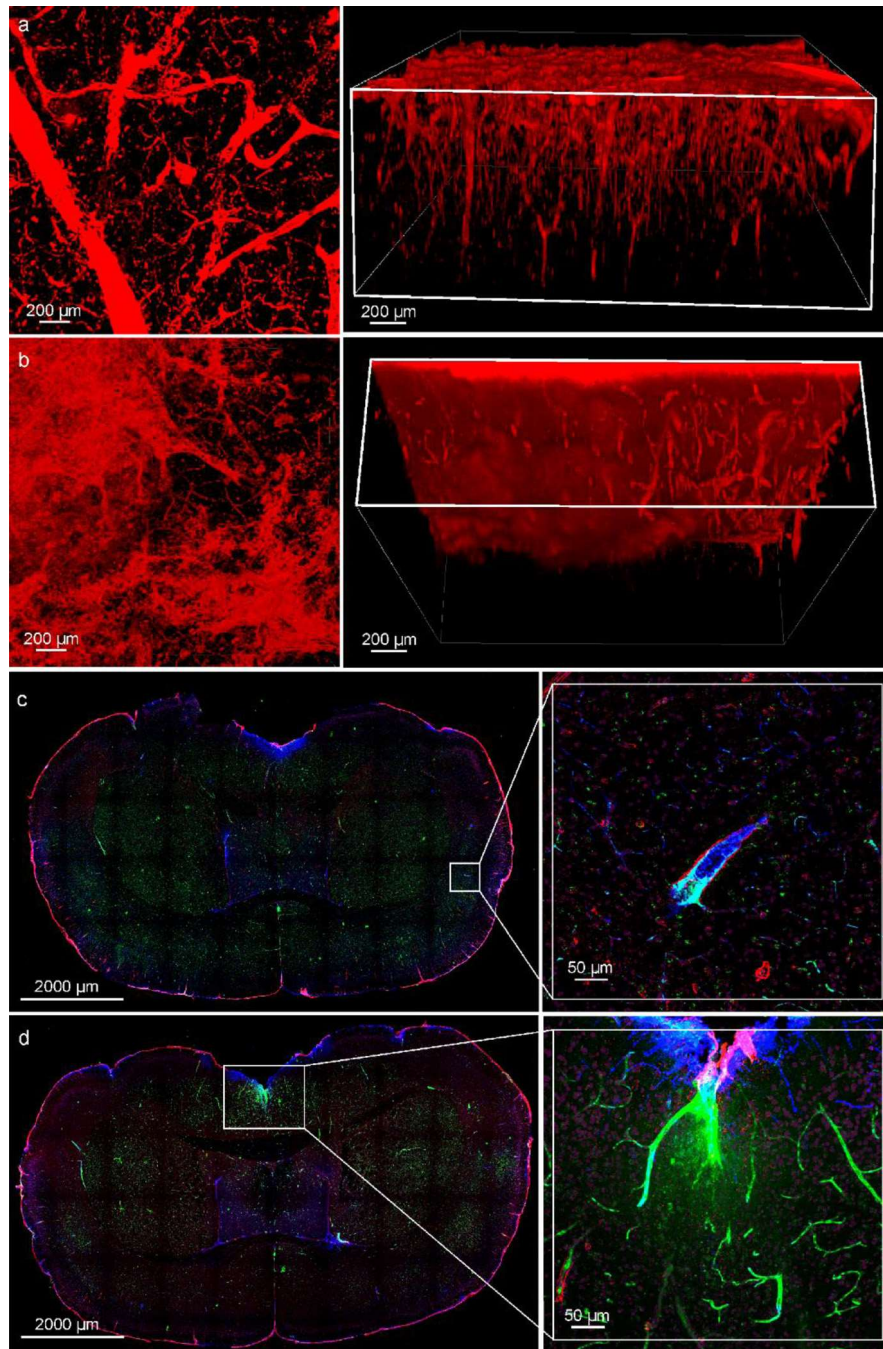


Fig. 3. The PDT-mediated OBBB: (a and b) Representative images (2D on the left and 3D on the right) of in vivo multiphoton analysis of the cortex before PDT (a) with the intact cerebral vessels and 1 h after PDT with 5-ALA (i.v.) when Evans Blue (red) leakage was observed as red clouds around the cerebral vessels; (c and d) Representative images of ex vivo confocal analysis of the cortex before PDT (c) and 1 h after PDT with 5-ALA (i.v.). The extravasation of Evans Blue (green) was observed as green clouds around the cerebral vessels marked with CD 31 (red), astocytes are marked with GFAP (blue).

4. Discussion

PDT is an innovative therapeutic method for treating brain tumors [1–7]. 5-ALA is PS, which is often used in this method [5–7]. It is generally accepted that PDT suppresses the growth of tumor cells due to the toxic effects of singlet oxygen generated by photo-excited PS. However, the effects of singlet oxygen are not selective for only malignant cells, as was previously thought. It has recently been discovered that PDT with intravenous injection of 5-ALA causes OBBB in healthy animals, i.e. singlet oxygen can increase the permeability of the cerebral vessels of intact brain tissues [8–14]. Furthermore, PDT with intracisternal injection of 5-ALA induces damages of MLVs leading to reducing brain's drainage [15–19]. Here, we aimed to answer the question whether PDT-mediated OBBB can lead to extravasation of 5-ALA into CSF and cause damage of MLVs in the same way as occurs with its intracisternal administration.

We clearly show that PDT with 5-ALA injected intravenously causes an increase permeability both the meningeal and cerebral blood vessels of healthy mice. These data suggest that the photo-excited 5-ALA can affect the intact endothelial cells of blood and lymphatic vasculature. Indeed, PDT with 5-ALA induces a disorganization of interaction between the tight junction (TJ) proteins, such as claudin-5, VE-cadherin and zonula occludens-1 playing an important role in regulation of the permeability of both the blood and lymphatic endothelium [13,26]. These changes are associated with an increase in the expression of molecular membrane factor (beta-arrestin-1, ARRB1), which can induce the internalization of TJ proteins leading to the loss of their surface in space between the endothelial cells, which can be one of the mechanisms of PDT-mediated OBBB [13,27]. So, Hebda et al. demonstrate the internalization of VE-cadherin as a one of the mechanisms of the BBB disruption [27]. PDT-related dysfunction of TJ proteins are accompanied by oxidative stress with a rise of the level of malondialdehyde and a decrease in oxygen in brain tissue [13]. It is important to note that PDT effects on the BBB integrity is reversible and are observed during 3-4 hrs after PDT application with further recovery of BBB [8–14].

In the next step, we aimed to study the dorsal MLV network covering the main venous sinuses (SSS and TS) before and after PDT-induced OBBB. Our results revealed that the LYVE-1 + coverage of SSS and TS dramatically reduced 7 days after PDT suggesting photo-damages of MLVs. Photoablation effects with intravenous administration of 5-ALA were less than those with its intracisternal injection, but statistically significant compared with the control. Thus, these results confirm our hypothesis that during PDT-induced OBBB, 5-ALA can leak from the meningeal blood vessels into CSF filling the meninges and, being photo-excited, affect the endothelium of MLVs. Rose Bengal is PS used for photothrombotic stroke, can also cause damages of MLVs due to photocoagulation of the endothelial cells of the meningeal blood vessels because the laser irradiation passes through the meninges before reaching the surface of the cortex [28].

Finally, we tested the drainage function of MLVs by observing lymphatic removal of FITCD from the brain to dcLNs before and after PDT-induced OBBB. With this aim, FITCD was injected into the right lateral ventricle. Afterward, FITCD was allowed to spread in CSF during 1.5 hrs and remove with the CSF flow into the peripheral lymphatics. Since, dcLNs are the first anatomical station for collection of CSF draining from the brain [29,30], we analyzed the intensity of accumulation of FITCD in dcLNs before and after PDT-related OBBB. The results demonstrate significant reducing lymphatic removal of FITCD from the brain in PDT-induced MLV-defected mice compared with the control groups (5-ALA i.c.m., 5-ALA i.v. or laser alone). Indeed, the fluorescent signal from FITCD was greater in dcLNs in the control groups vs. the PDT group suggesting effective clearance of fluorescent dye from brain tissues and its faster accumulation in dcLNs in mice with the intact MLVs than in mice with photo-damaged MLVs.

5. Conclusion

In summary, the results of this study revealed the new vascular effects of PDT with 5-ALA. It was found that upon photoexcitation, 5-ALA induces an increase in the permeability of both the cerebral and meningeal blood vessels. As was established in our previous studies, this is associated with a change in the expression of the TJ proteins and the development of oxidative stress [13,26]. PDT-induced increase in the permeability of the meningeal blood vessels leads to the extravasation of photo-excited 5-ALA from the bloodstream into the meninges, where MLVs are located. This, in turn, provokes photo-damage of the endothelium of MLVs, presumably by the same mechanism that PDT causes damage to the blood endothelium. Indeed, the same TJ proteins are expressed in both the lymphatic and blood endothelium, which suggests the similarity of the processes of PDT-induced vascular photo-damage. The PDT-induced damage of MLVs leads to a dramatic reduction of brain's drainage that can be accompanied by excessive accumulation of fluids in brain tissues. The PDT-induced damaging effects on MLVs is important to consider in the PDT therapy for brain diseases as a possible side effect of PDT with 5-ALA. These results provide an important informative platform for a better understanding of the vascular effects of PDT, a reassessment of advantage and disadvantage of this method and optimization of clinical practice guidelines for the save use of PDT in the therapy of brain tumors and other brain pathologies.

Funding. Russian Science Foundation (23-75-30001).

Acknowledgments. The preparation of the brain tissues, immunofluorescence analysis for optical imaging were carried out using equipment of the research center "SYMBIOSIS" within project No. GR 1022040700963-8 (IBPPM RAS). **Author Contributions.** O.S.-G. initiated and supervised this work. A.T., A.D., A.D., A.S. and I.F. prepared samples and performed the confocal analysis; M.Z. made the statistical analysis; A.E., V.A., D.Z., M.T., M.M., V.K., E.I., D.M., D.T., S.P. performed most of the experiments. O.S.-G. and I.B. reviewed all results and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Disclosures. The authors declare no conflict of interest.

Data availability. The data that support the findings of this study are available on request from the corresponding author.

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