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Photodynamic opening of blood-brain barrier

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Abstract: Photodynamic treatment (PDT) causes a significant increase in the permeability of the blood-brain barrier (BBB) in healthy mice. Using different doses of laser radiation (635 nm, 10-40 J/cm^2) and photosensitizer (5-aminolevulinic acid – 5-ALA, 20 and 80 mg/kg, i.v.), we found that the optimal PDT for the reversible opening of the BBB is 15 J/cm^2 and 5-ALA, 20 mg/kg, exhibiting brain tissues recovery 3 days after PDT. Further increases in the laser radiation or 5-ALA doses have no amplifying effect on the BBB permeability, but are associated with severe damage of brain tissues. These results can be an informative platform for further studies of new strategies in brain drug delivery and for better understanding of mechanisms underlying cerebrovascular effects of PDT-related fluorescence guided resection of brain tumor.

OCIS codes: (170.0170) Medical optics and biotechnology; (170.3880) Medical and biological imaging; (170.1470) Blood or tissue constituent monitoring; (170.2655) Functional monitoring and imaging.

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1. Introduction

The blood-brain barrier (BBB) is a gatekeeper located on the endothelial cells of the microvasculature and controls the passage of blood-borne agents into the brain tissues playing

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an important role for the health of the central nervous system. These protective mechanisms restrict the entrance of many drugs into the brain. There are 7000 drugs, which are registered in the Comprehensive Medicinal Chemistry database but only 5% of them are available to treat the neuronal diseases because antibodies, recombinant proteins, therapeutic genes and most small molecules simply cannot penetrate the BBB [1, 2]. This is the reason why approaches to open the BBB have received significant research attention from around the world in the last four decades. There are more than 70 different physical, chemical and biological methods for overcoming of the BBB [3–5]. However, no methods are developed yet, which are widely used in daily clinical practice for the opening of the BBB due to their invasiveness and challenging nature [6–8].

Photodynamic therapy (PDT) is a treatment that combines light irradiation and photosensitizing agents (porphyrins, chlorins and many other photodynamic dyes). The excited photosensitizer directly oxidizes biomolecules (Type I) and/or interacts with molecular triplet oxygen ($^3O_2$) producing singlet oxygen ($^1O_2$) (Type II) causing cancer cells apoptosis and/or necrosis through plasma and mitochondria membrane disrupture.

5-aminole-vulinic acid (5-ALA) is a pro-drug that has been used in a variety of PDT applications due to its safety and suitability for oral administration [9,10]. 5-ALA itself does not produce fluorescence. After being administrated, 5-ALA is metabolized in the cells to protoporphyrin IX that accumulates in tumor tissues, exhibiting a photodynamic effect via light irradiation of the targeted tissue and allowing the neurosurgeon to clearly see and accurately resect the tumor [9,11]. The anti-cancer effect of PDT in this case is based on its induced damage of the endothelial cells, resulting in tumor microvasculature collapse [12,13].

It has recently been shown in several studies that PDT can temporally increase the BBB permeability. Hirschberg et al. using a 400 μm bare flat-end quartz fiber (635 nm) and stereotaxic procedure show targeted opening of the BBB for gadolinium [14]. Madsen et al. using 670 nm laser application through the skull and aluminum phthalocyanine disulfonate ($AlPcS_{2a}$) demonstrate the opening of the BBB for macrophages [15–17].

Thus, a new application of PDT to increase permeability of the BBB to small weight molecules and immune cells using for magnetic resonance imaging (MRI) was shown for the first time.

In this research, we studied the PDT-related BBB disruption using the widely implicated in medicine PDT approach. We employed a 635 nm laser and 5-ALA for fluorescence guided resection of glioblastoma with the aim of better understating the mechanisms underlying vascular collapse by PDT and its effectiveness as a potential method of brain drug delivery. We also analyzed the BBB permeability to high weight molecular substances followed PDT using different light doses and concentrations of 5-ALA.

2. Methods

2.1. Subjects

Experiments were carried out in mongrel mice weight 20 g (n = 103). The animals were divided into five groups: 1) intact, without laser irradiation (the control group); 2-5 included mice after laser irradiation with different laser doses: 2) 10 J/cm^2; 3) 15 J/cm^2; 4) 20 J/cm^2; 5) 40 J/cm^2. All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals". The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Saratov State University (Protocol H-147, 17.04.2001).

2.2. PD treatment protocol

The mice were anaesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg, i.p.), and fixed in a stereotactic frame. The PDT was performed 30 min after intravenous injection of 5-ALA (20 mg/kg). The epicranium was exposed by a parieto-occipital midline skin incision.

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With the use of microsurgical techniques, the periosteum was pushed back and a biparietal parasagittal groove-shaped trephination (2 × 2 mm) was performed with a microdrill (Mikroton, Aesculap) during continuous irrigation with saline to prevent heating of the tissue. Special care was taken to prevent penetration of the dura mater.

A continuous wave (CW) light-emitting device with controlled power fluence measured on the surface of a 8 mm diameter solid light guide in the range of 20 - 200 mW/cm^2 was made on the base of a high power red LED (XPeBRD-L1-0000-00901, CREE, Inc.), which emits a 1W maximum light power at 635 nm. The spectral width of emitted light at half peak of intensity was 30 nm. Light from this 635 nm diode emitter was delivered to the mice brains, with the mice undergoing irradiation using two regimes of light exposure, achieving several various light doses 10, 15, 20 and 40 J/cm^2 using irradiation at constant power fluence at 40, 40, 60, 100 mW/cm^2 and irradiation times of 250, 375, 333 and 400 seconds, respectively. The effect of laser brain tissue heating was controlled by a Pico USB TC-08 thermocouple data logger (USA). The temperature did not exceed 3-4° C above the mouse body level of 28° C for the highest irradiation dose.

2.3. Assessment of the BBB permeability

To evaluate the BBB permeability we used three different methods.

The first test was a spectrofluorometric assay of Evans Blue dye (EBd) extravasation. The EBd (Sigma Chemical Co., St. Louis, MI, USA) was injected in a single bolus dose (2 mg/25 g mouse, 1% solution in physiological 0.9% saline) into the right femoral vein. At the end of circulation time (30 min), mice were decapitated, their brains were rapidly collected and placed on ice. The EBd extraction and visualization was performed accordingly to Wang et al. [18]. The analysis of EBd extravasation was performed in groups 1 – 5, n = 7 for each group.

The second test was confocal microscopy of dextran extravasation [19]. In brief, FITC-dextran 70 kDa (Sigma) was injected intravenously (4 mg/25 g mouse, 0.5% solution in 0.9% physiological saline) and circulated for 2 min. Afterwards, mice were decapitated and their brains rapidly removed and fixed in 4% paraformaldehyde (PFA) for 24 hrs. These brains were cut into 50-µm thick slices on a vibratome (Leica VT 1000S Microsystem, Germany) and analyzed on a confocal microscope (Olympus FV10i-W, Olympus, Japan). Approximately 8–12 slices per animal from cortical and subcortical (excepting hypothalamus and choroid plexus where the BBB is leaky) regions were imaged. The confocal microscopy of the BBB permeability was performed in groups: The analysis of EBd extravasation was performed in groups 1 – 5, n = 8 for each group.

The third test was histological analysis of the BBB permeability to solutes. This study was performed in the same groups, which were used for confocal microscopy assessment. All mice were decapitated after the performed experiments. The samples were fixed in 10% buffered neutral formalin. The formalin fixed specimens were embedded in paraffin, sectioned (4 µm) and stained with haematoxylin and eosin. The histological sections were evaluated by light microscopy using the digital image analysis system Mikrovizor medical µVizo-103 (LOMO, Russia).

2.4. Statistical analysis

The results were reported as mean ± standard error (SE). The differences from the initial level in the same group were evaluated by the Wilcoxon test. Intergroup differences were evaluated using the Mann-Whitney test and ANOVA-2 (post hoc analysis with the Duncan's rank test). Significance levels were set at $p < 0.05$ for all analyses.

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3. Results and discussion

3.1. PDT- induced BBB disruption evaluated by assessment of EBd leakage

At the first step of our work, we studied the PDT-related increase in the BBB permeability to intravenously injected EBd, a classical test of BBB leakage to high weight molecular molecules [20]. EBd is a 961 Da dye that binds to serum albumin and makes EBd in blood into a high molecular weight complex (68.5 kDa), which cannot permeate the intact BBB. Therefore, EBd leakage into the brain tissues indicates BBB disruption [21, 22].

How do different light doses and concentrations of photosensitizer affect the BBB function?

To answer to this question, we analyzed four light doses: 10, 15, 20, 40 J/cm^2 and two 5-ALA doses – 20 mg/kg, which is effective for fluorescence-guided resections of malignant gliomas [9] and a higher dose (80 mg/kg).

Table 1. The 5-ALA-PDT-related changes in the BBB permeability to Evans Blue

Light doses	Content of Evans Blue in the brain (μg/g tissue)	
No irradiation (control group, n = 7)	0.52 ± 0.03	
	90 min after light irradiation	**4h after light irradiation**
10 J/cm^2, 40 mV, 250 sec (n = 7)	9.90 ± 0.08 *	0.73 ± 0.05
15 J/cm^2, 40 mV, 375 sec (n = 7)	17.33 ± 0.09 *	0.62 ± 0.09
20 J/cm^2, 60 mV, 333 sec (n = 7)	17.91 ± 0.08 *	0.64 ± 0.04
40 J/cm^2, 100 mV, 400 sec (n = 7)	17.87 ± 0.09 *	0.58 ± 0.05

$p<0.05$: *- vs. before irradiation

During the first stage of experiments, we used the clinically recommended dose of 5-ALA – 20 mg/kg. The BBB permeability to EBd was analyzed every 30 min after 5ALA-PDT, over a 4h duration. Here we presented two time points - 90 min after PDT, when we observed the first significant changes in the BBB and 4h after PDT, when we did not see further increases to the leakage of EBd.

The data presented in Table 1 show that the illumination dose of 10 J/cm^2 caused a 19.0 fold increase in the BBB permeability to EBd compared to initial state (without PDT). A light dose of 15 J/cm^2 was accompanied by a 33.3 fold increase in the leakage of EBd, which was 1.7 higher than with an irradiation dose of 10 J/cm^2. Further increase in light dose was not associated with more increase in the level of EBd in the brain tissue, i.e. the application of higher irradiation doses (20 and 40 J/cm^2) caused similar effects on the BBB as irradiation dose of 15 J/cm^2 did.

No effect on the BBB was observed if laser irradiation (10, 15, 20, 40 J/cm^2, n = 7 in each group) was applied alone (without 5-ALA).

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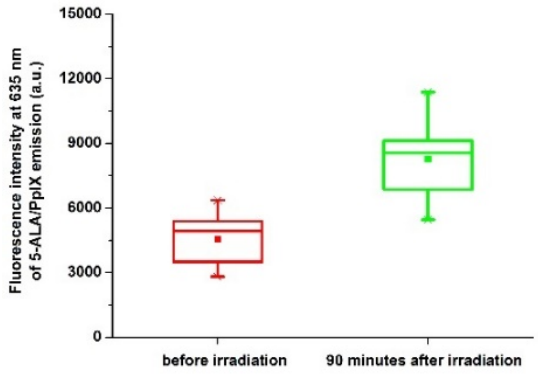

Fig. 1. Fluorescent intensity at 635 nm associated with 5-ALA signal before light irradiation and 90 min after light irradiation of mouse brain.

Figure 1 shows fluorescent intensity before light irradiation and 90 min after light irradiation, when the BBB was opened. Figure 2 shows fluorescent intensity at 635 nm with effect of physiological and high dose of 5-ALA on mouse brain.

In the next step of our experiments, we analyzed the effect of 5-ALA in high doses (80 mg/kg, n = 8) on the BBB permeability to EBd. The light dose – 15 J/cm^2 that was optimal for the opening of the BBB when using a 20 mg/kg dose of 5-ALA was selected.

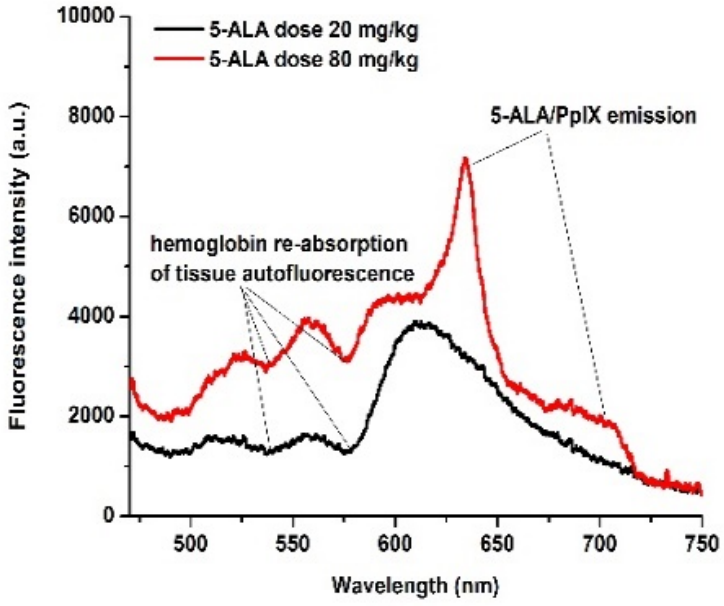

Fig. 2. Fluorescence spectra of brain tissues with different 5-ALA dose applied – autofluorescence and exogenous fluorophore emission are observed.

The results showed that the high dose of 5-ALA caused a 75.6 fold greater increase in the leakage of EBd vs. the control group (39.81 ± 0.01 vs. 0.52 ± 0.03, $p<0.05$), which was 2.2 times higher compared to the 20 mg/kg dose of 5-ALA (39.81 ± 0.01 vs 17.33 ± 0.09,

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$p<0.05$). None of the groups exhibited effects on the brain temperature from laser illumination.

3.2. PDT-induced BBB disruption evaluated by assessment of dextran (70kD) and solutes leakage

In this series of experiments, we tested the BBB stability after PDT, studying dextran extravasation (confocal imaging) and solutes leakage (histological analysis) (Fig. 3). The dextran extravasation was determined as clear specific fluorescence occurring outside of the vessel walls and was classified as weak (+), medium (+ +), strong (+ + +), and diffusive (+ + + +) [19]. Weak infiltration was defined for a faint cloud, clearly associated with one vessel site of the tracer leakage. Medium was defined by a bright cloud, clearly associated with one vessel site of the tracer leakage. Strong was defined as a bright cloud, not clearly associated with one vessel leakage, but a group of vessels. Diffusive was defined as extensive leakage of the tracer without clear association with specific vessels. Taking into account the above results, we studied the BBB disruption 90 min after 5-ALA-PDT influence.

Our results showed that laser irradiation doses from 10 to 40 J/cm^2 with 5-ALA (20 mg/kg, i.v.) was associated with strong leakage of dextran from cerebral capillaries resulting in formation of red clouds around microvessels.

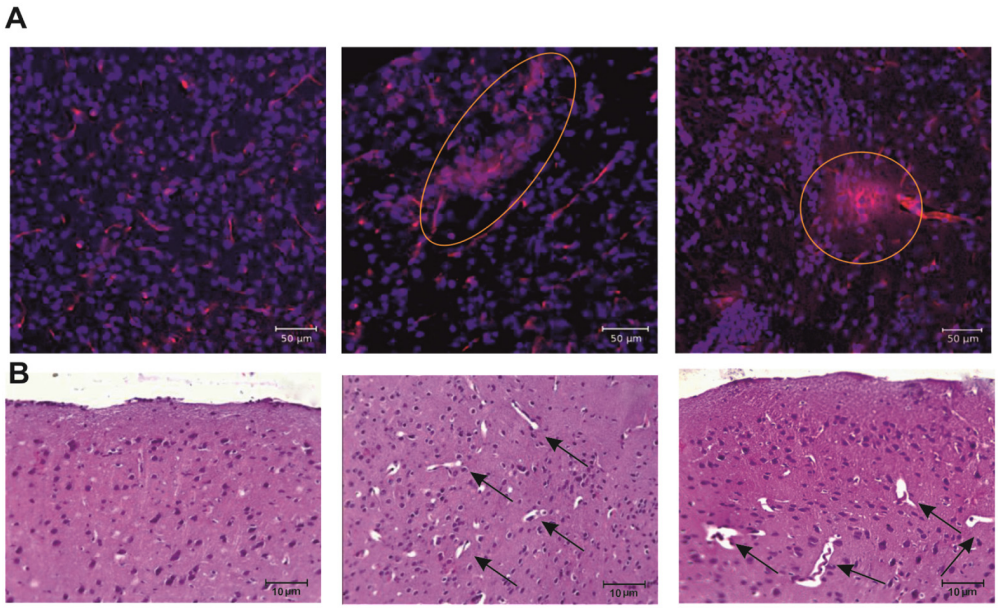

Fig. 3. The PDT-induced BBB disruption evaluated by confocal imaging of FITC-dextran (70 kD) extravasation from cerebral microvessels into the brain parenchyma and histological analysis of solutes leakage. A – confocal imaging: left – no extravasation of FITC-dextran in control group without laser application; middle- strong extravasation of FITC-dextran defined as a bright cloud (circled) clearly associated with group of vessels [19]; right - diffuse extravasation of FITC-dextran (circled) defined as extensive leakage of the tracer including several groups of cerebral microvessels. B – histological results: left – no solutes leakage in normal brain tissues; middle – moderate perivascular edema suggesting solute leakage from cerebral microvessels and accumulation of solutes in perivascular space; right – severe perivascular edema reflecting the stronger BBB disruption for solute leakage.

The high dose of 5-ALA (80 mg/kg, i.v., laser dose - 15 J/cm^2) was accompanied by diffuse type of dextran extravasation, i.e. higher BBB disruption.

Histological data demonstrated similar tendency comparing to the confocal imaging of the brain slices. Indeed, laser doses from 10 to 40 J/cm^2 with 5-ALA (20 mg/kg, i.v.) caused

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moderate perivascular edema suggesting solute leakage from microvessels. High dose of 5-ALA induced more extensive perivascular edema with highly intensive accumulation of solutes in the perivascular space.

The complete recovery of brain tissues after PDT-induced perivascular edema due to the opening of the BBB to solutes was observed 3 days later (histological data, not presented).

Thus, our results suggest that PDT (λ = 635 nm + 5-ALA) causes temporal increase in the BBB permeability to EBd (68.5 kDa) and dextran (70 kDa) mimicking the BBB leakage to high weight molecular molecules such as proteins [19]. These effects depend on irradiation dose and concentration of photosensitizer. The optimal laser doses that induced the opening of the BBB were 10-15 J/cm^2. Interestingly, further increasing the light dose to 40 J/cm^2 was not accompanied by more extensive changes in the BBB. This is probably due to the protective mechanisms via re-distribution of cerebral blood flow that we observed after sound-induced opening of the BBB [23].

The concentration of 5-ALA (20 mg/kg, i.v.) was adapted to mice from human data [9]. We estimated that 5-ALA in this dose caused a moderate effect on the BBB and brain vessels, while a high dose of 5-ALA (80 mg/kg, iv) was associated with significant BBB and brain injuries.

Our data are consistent with others who also show dependence of BBB disruption on light doses [14–17]. Hirschberg et al. using stereotaxic PDT in healthy rats (632 nm + high dose of 5-ALA 125 mg/kg, i.p.) demonstrated the increase in BBB permeability to low molecular weight gadolinium 2h following PDT and the recovery of the BBB 72h later at the lowest fluence level (9J/cm^2) [14]. In contrast, high irradiation doses (17 and 26 J/cm^2) were accompanied by longer recovery of the BBB function (2 weeks) and serious damages of brain (necrosis, hemorrhage). Madsen et al. showed that PDT-related opening of the BBB is useful for macrophage transportation through the BBB using LED irradiation (670 nm, 2.5 J/cm^2) and photosensitizer ($AlPcS_{2a}$: 1 mg/kg, i.p.) through the skull in healthy rats [15].

In our work, no effect on the BBB was observed if irradiation was given in the absence of 5-ALA, which was in agreement with results of others [14].

The mechanisms underlying PDT-mediated opening of the BBB are poorly understood. There is evidence that PDT has a direct effect on the endothelial cells, through tight junctions increasing the gaps between endothelial cells via changes of the cytoskeleton, cell rounding and constriction loss due to microtubule depolarization [24–27]. The PDT-induced singlet oxygen ($^1O_2$) generation causes an imbalance of endothelial regulation of vascular relaxation, which is an important mechanisms underlying the decreasing of vascular resistance to photo-induced oxidative stress, leading to the redistribution of calcium, reduction of influx and release of calcium from intracellular stores [28–34]. The $^1O_2$-mediated depletion of calcium stores is thought to be the crucial event in mechanisms underlying direct effects of $^1O_2$ during vasoconstriction and vasorelaxation.

4. Conclusion

Our results on healthy mice clearly show that small doses of PDT (635 nm, 15 J/cm^2) with a concentration of 5-ALA, 20 mg/kg (i.v.) is optimal for the reversible BBB opening for high weight molecular substances such as EBd (68 kDa) and dextran (70 kDa). Further increase in light doses has no amplifying effect on the BBB. The increasing of 5-ALA concentration is accompanied by severe disruption of the BBB resulting in injuries of brain tissues (perivascular edema). We believe that these results are of high importance for the deeper understanding of PDT effects on the cerebral vasculature and its further applications in drug brain delivery.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.