INTRODUCTION

Blood-brain barrier (BBB) permeability is altered in various pathological states, including ischemic stroke. Apart from being an important mechanism of secondary brain injury, BBB disruption with plasma protein extravasation was implied in late consequences of acute cerebral insults, namely epileptogenesis (1-4). The extent of BBB disruption differs among individual animal models of focal cerebral ischemia (5-9). Many factors contribute to this variability, including the choice of species, its vascular anatomy, capacity of collateral circulation, the duration of ischemia (transitory or permanent), the means of producing ischemia (large vessel occlusion, focal vasoconstriction, or microvascular occlusion, e.g. photothermal lesion), and, possibly, the choice of anesthesia. Because BBB disruption in some models follows a biphasic course (10), the timing of evaluation becomes a major issue as well.

Usually, investigators focus on BBB disruption in the perilesional tissue. However, focal ischemia was shown to induce changes in the cerebral blood flow, brain metabolism and excitability also in remote functionally connected areas - a concept known as diascisis (11-14). The possibility that diascisis could include remote changes in BBB permeability was not tested.

In the present study, we evaluated perifocal, as well as contralateral changes in BBB permeability and tested the influence of two anesthetics on the results. Adult Wistar rats were randomly anesthetized with pentobarbital (PB) or ketamine-xylazine (KX). Rats received intravenously (i.v.) Rose Bengal followed by Evans Blue (EB). Stereotactically defined spots on denuded skull were irradiated by laser (532 nm) for 18 min. Twenty four hours later, rats were killed, brains perfused, fixed, sectioned and slices analyzed by fluorescence microscopy. Volume of necrosis and volume of EB-albumin extravasation were calculated. Evidence of BBB breakdown in remote brain areas was sought and compared to sham handled controls. BBB disruption was consistently present, frequently with EB-albumin accumulating cells. Total lesion volume did not significantly differ among groups (TLVPB=9.4±1.3 mm³ vs. TLVKX=8.3±2.1 mm³); same was true for the volume of necrosis (NV_P=5.1±0.7 mm³ vs. NV_KX=6.3±1.9 mm³). However, volume of EB-albumin extravasation area was significantly smaller in KX group (EBEV_PW=4.3±0.8 mm³ vs. EBEV_KX=2.0±0.5 mm³; p=0.0293). Median background EB-fluorescence signal density was higher in PB group (p<0.0001). Furthermore, regional increase in EB-fluorescence was found in two animals in PB group. Our study shows that anesthesia with NMDA-antagonist ketamine and α2-adrenergic agonist xylazine may reduce BBB breakdown in photothermal lesion. Pentobarbital anesthesia lead to increased BBB permeability in the contralateral hemisphere.

Key words: anesthesia, blood-brain barrier, cerebral ischemia, ketamine, pentobarbital, photothermal lesion, stroke
Experimental groups and anesthesia

Sixteen rats were randomly assigned to two experimental groups. Pentobarbital (20 mg/kg i.p.; Sigma, Czech Republic) was used for anesthesia in the first group (“PB”; N=8), a mixture of ketamine and xylazine (ketamine 80 mg/kg-xylazine 7 mg/kg i.p.; Sigma, Czech Republic) in the latter (“KX”; N=8). For comparison of background fluorescence signals, a sham-handled control group was created (N=4). Depth of anesthesia was tested at regular 5 min intervals by pressing the skin of the animal’s hind-limb sole with anatomical tweezers. In case of hind-limb flexion, additional intrapertoneal bolus of half the calculated dose of ketamine was given to ensure surgical anesthesia. Usually, one additional dose of anaesthetic was needed prior to fixation of the animal in the stereotactic frame (approx. 20 minutes after the induction of anesthesia). Throughout the experiment, the animals did not show signs of respiratory distress, or other deficits in vital signs.

Surgery and photothrombosis

Photothrombosis was performed as previously described (15). After the induction of anesthesia, the scalp of the head was incised (2 cm length in midline) and the skull overlaying the left sensorimotor cortex was cleaned from soft tissues. A bolus of photosensitive dye Rose Bengal (“RB“-Sigma, Czech Republic; 20 mg/2 ml/kg, dissolved in 0.9% NaCl) was applied slowly into the systemic circulation via tail vein, followed by a bolus of Evans Blue (“EB“-Sigma, Czech Republic; 0.04 g/kg/2 ml; dissolved in 0.9% NaCl). After the application of dyes, the animals were positioned in a stereotactic frame. Next, three stereotactically defined adjacent points on the scull overlaying the hind-limb area of the left sensorimotor cortex were irradiated by a diode laser beam (532 nm; power density 50 mW/mm²; illuminated area<1 mm²). Each point was irradiated for 6 min by a diode laser beam (532 nm; power density 50 mW/mm²; illuminated area<1 mm²). Each point was irradiated for 6 min. After the end of photothrombosis, the animals were left to recover for 24 hours. In contrast to other tracers (e.g. natrium fluorescein), EB is almost completely bound to serum albumin. Thus, EB extravasation is a marker of albumin transport outside the blood vessels. We chose to apply EB prior to photothrombosis to mirror any change in BBB permeability within the first 24 hours of ischemia (from the onset of photothrombosis to brain perfusion and fixation).

Histology, image processing and evaluation

Twenty-four hours after photothrombosis, in deep urethane anesthesia, all animals were transcardially perfused with a solution of paraformaldehyde and decapitated. Brains were removed, fixed in paraformaldehyde and sectioned into 40 µm coronary slices. Lesion dimensions and the distribution of red fluorescence signal emitted by EB-albumin complex in green light were then studied with a fluorescence microscope (Olympus®). Digital microphotographs of all slices (both ipsilateral and contralateral cortex) were obtained at fixed image acquisition parameters (magnification, exposition, sensitivity, resolution, image format). Additional higher power microphotographs were acquired as needed. All digital photographs were then analyzed with a freely available utility ImageJ 1.37v (Wayne Rasband, National Institute of Health, USA; http://rsb.info.nih.gov/ij/).

1. Ischemic lesions and perifocal blood-brain barrier disruption

Area of necrosis was clearly delimited on each section (Fig. 3). With ImageJ tools, the area of necrosis was measured on each slice and multiplied by the thickness of the section – 40 µm. The sum of values from individual slices gave the volume of necrosis (NV) in an individual animal. The volume of EB-albumin extravasation (volume of BBB disruption) was measured similarly. The area of EB-albumin extravasation was delineated manually on each slide. Its border was defined as the point where clear increase in fluorescence signal was not anymore observable (Fig 3A). Again, the sum of values from individual slices gave the volume of EB-albumin extravasation (EBEV) in an individual animal. All measurements (608 slices in the PB group, 546 slices in the KX group) were performed by a single investigator (DK) to avoid inter-rater variability.

2. Remote blood-brain barrier disruption

BBB disruption in the contralateral hemisphere was evaluated by two methods. Firstly, all slices were visually examined for regions of increased fluorescence signal and for the occurrence of EB-positive cells (i.e. cells accumulating EB-
Secondly, we measured differences in background EB-fluorescence signal between the groups. In every sixth contralateral section, a virtual frame of $1 \times 2$ mm was placed on the contralateral cerebral cortex. The frame was consistently positioned in a "mirror" region corresponding to the contralateral area of ischemic lesion. Mean gray value (mean signal density) was measured within this region by ImageJ tools (mean gray value = the sum of the gray values of all the pixels in the selection divided by the number of pixels; in RGB images, each pixel was converted to gray-scale using the formula: gray = (red + green + blue) / 3). Thus, we have obtained a set of mean gray values (mean signal densities) in all groups (PB, KX, controls). Differences between the groups were then statistically evaluated.

Fig. 3. Fluorescence microscopy images of the lesions. (A) Typical photochemically induced cortical ischemic lesion. Necrotic core can be seen with a surrounding brightly red area of extravasated EB-albumin. Lines denote the borders of necrotic core and the area of BBB disruption. The brightly red material (EB-albumin) in the cortical blood vessels can be seen in the lower half of the image. (B) A detailed view of the rim of necrosis and area of BBB failure. EB-albumin accumulating cells can be seen as bright red dots with a surrounding halo of EB-albumin extravasation (black arrowheads). (C) A larger blood vessel with EB-albumin adhering to its walls and escaping into the extracellular space (black arrowheads). (D) A detail view of the rim of the necrosis with EB-albumin accumulating cells and a blood vessel extending to the area of blood brain barrier alteration (black arrowhead). The extravasation of EB along the blood vessel walls is evident. (E) Low power image of the hemisphere contralateral to ischemic lesion (animal anesthetized with PB). A change in the background signal in the left half of the hemisphere (representing change in blood-brain-barrier permeability) can be noted (black arrowhead). (F) Signal changes in the medial part of the hemisphere contralateral to ischemic lesion (animal anesthetized with PB) (black arrowheads).
Statistical evaluation

GraphPad Prism 5.01 (GraphPad Software, Inc.) was used for statistical evaluation of the results. Normal distribution of the results was tested by D’Agostino & Pearson omnibus normality test. Statistical significance of differences between PB and KX group with respect to volume of necrosis and volume of EB-albumin extravasation were evaluated by unpaired t-test. Mean signal density values in the contralateral hemisphere were not normally distributed. Therefore, non-parametrical tests (Kruskal-Wallis test, and Mann-Whitney U test) were used to evaluate differences in this parameter among the groups.

RESULTS

Ischemic lesions with central area of necrosis were observed in all animals of both experimental groups 24 hours after photothrombosis (Fig. 3A). As a rule, a large area of EB-albumin extravasation surrounded the lesions, extending sometimes to the corpus callosum and subcortical structures. In some slices, brightly red EB-albumin accumulating cells were observed (Fig. 3B-arrowhead). Clusters of these cells were usually found at the border of the necrotic core and the area of BBB breakdown (Fig. 3B, 3D). EB-stained material was found in the blood vessels both in the ipsilateral and contralateral hemisphere and in subcortical structures (see Fig. 3C. 3D-arrowhead). Pial blood vessels, as well as perforating blood vessels perpendicular to the pial surface were sometimes found to be lined with EB-albumin complex. In larger vessels, EB-albumin complex was found adhering to the vessel wall and escaping into the extracellular space (Fig. 3C, 3D-arrowhead).

Total volume of lesion (TLV; i.e. volume of necrosis + volume of EB-albumin extravasation) did not significantly differ between the groups (TLV<sub>PB</sub>=9.4±1.3 mm<sup>3</sup> vs. TLV<sub>KX</sub>=8.3±2.1 mm<sup>3</sup> ). The volume of necrosis (NV) was slightly larger in the ketamine-xylazine group (NV<sub>PB</sub>=5.1±0.7 mm<sup>3</sup> vs. NV<sub>KX</sub>=6.3±1.9 mm<sup>3</sup> ), however, the difference was not statistically significant. On the other hand, the volume of EB-albumin extravasation (EBEV) was significantly smaller in the KX group (EBEV<sub>PB</sub>=4.3±0.8 mm<sup>3</sup> vs. EBEV<sub>KX</sub>=2.0±0.5 mm<sup>3</sup>; p=0.0293, two-sided unpaired t-test) (see Fig. 1). Median EB-fluorescence signal density in the hemisphere contralateral to ischemic lesion was significantly increased in PB group (p<0.0001, Mann-Whitney U test). Median EB-fluorescence signal density in the KX group was similar to the control group (Fig. 2). Moreover, in two animals from the pentobarbital group, diffuse changes in EB-fluorescence signal intensity were found in remote areas in the contralateral hemisphere (Fig. 3E, 3F-arrowheads). Unequivocal EB-albumin accumulating cells were not found in the contralateral cortex.

DISCUSSION

The extent and anatomical pattern of BBB disruption differs among experimental models of focal ischemia. Breakdown of BBB to proteins is invariably present in transient middle cerebral artery occlusion (t-MCAO) (16-18), whereas it is not a typical feature of permanent occlusion model (p-MCAO) (6). Extensive perifocal vascular leakage is an important feature of photobothrombosis (8), and it was shown to persist at least 24 hours after laser irradiation (19). In agreement with these findings, we have consistently observed areas of increased EB-albumin fluorescence surrounding necrotic core of ischemic lesions in the irradiated area. Areas of increased EB-fluorescence extended into the cerebral white matter and corpus callosum. Within these regions, we observed adherence of EB-positive material to the walls of larger penetrating vessels and its leakage into the extracellular space (Fig. 3A-D). Apart from diffuse staining of the parenchyma surrounding the lesion core, we have observed uptake of EB-albumin complex into cells (Fig. 3B, 3D-arrowheads). Plasma protein uptake into brain cells was described in various models of focal brain ischemia, as well as other insults (20-25) and may play an important role in epileptogenesis (2-4). Interestingly, animals subject to photobothrombosis (where large BBB breakdown is typical) frequently develop seizures (26-27).

Our study demonstrates that the extent of BBB disruption in the photobothrombotic model can be significantly reduced when a combination of NMDA antagonist ketamine and α2-adrenoceptor agonist xylazine is used for anesthesia, in contrast to GABA<sub>A</sub>-agonist pentobarbital. Similar results were observed when selective NMDA antagonist MK-801 was used in the t-MCAO model (28). We have found no direct evidence in the literature for xylazine-mediated alteration of blood-brain barrier permeability, although, in one study, a decrease of ethanol-induced BBB opening was observed following pretreatment with another α2-adrenoceptor agonist clonidine (29). On the other hand, the evidence for NMDA receptor mediated changes to BBB permeability is quite extensive (apart from other important pathophysiological roles of NMDA receptors, e.g. in excitotoxicity (30), memory consolidation, and mood disorders (31). NMDA stimulated uptake of HRP on isolated rat cerebral cappilaries (32), NMDA antagonist Hu-211 protects against BBB disruption in photobothrombosis (33), intrastriatal injection of NMDA induced extravasation of Lucifer yellow and this extravasation was prevented by NMDA antagonist (34) and NMDA applied topically on the cortex increased BBB permeability (35). Nevertheless, it must be noted, that in some models, this effect of NMDA receptor blockade on BBB permeability was not reproduced (36). Although we cannot rule out the role of xylazine in producing the observed effects, we suggest that the presented evidence favors the main role of ketamine in the observed results. The mechanism of NMDA-receptor mediated regulation of BBB permeability remains to be elucidated. Other investigators observed glutamate-induced changes in expression, phosphorylation and distribution of tight-junction proteins, such as occludin (37). Increased transcellular transport of EB-albumin complex in a receptor mediated fashion is also possible (4).

Apart from evaluating perilesional changes in BBB integrity, we also tested whether focal ischemic lesion can induce BBB alteration in remote (but functionally connected) areas (diaschisis). The median background EB-fluorescence signal density was significantly increased in the pentobarbital group (Fig. 7), compared to KX and controls. Furthermore, in two animals from the PB group, we have found regional increase in EB-fluorescence signal density in the medial part of the contralateral hemisphere (Fig. 3E, 3F-arrowheads). Also, in our preliminary experiments in animals anesthetized with PB, we have observed occasional cellular uptake of EB-albumin in the cerebellum. Although limited, these findings may support the possibility that the concept of diaschisis is also relevant to the regulation of permeability of blood-brain barrier and that alteration of neurotransmission may be involved.

The main limitation of our study is the absence of blood pressure monitoring throughout the experiment. However, with the intraperitoneal route of administration, the risk of hypotension is probably lower than with i.v. application. Moreover, episodes of hypotension would probably tend to influence the results in favor of pentobarbital as in the work by Chi et al. (38), which was not the case in our study.
In summary, our study shows that anesthesia with NMDA receptor antagonist ketamine decreases the extent of BBB breakdown in cortical photothrombosis. Furthermore, our results indicate that alteration of blood-brain barrier at sites contralateral to photochemically induced ischemic lesion is possible and that it may also be related to the choice of anesthesia. Further studies are needed to broaden these potentially clinically relevant observations.

Acknowledgements: The work was supported by following grants: Charles University Pregue, research project UNCE204010; Charles University, Prague, 264706/SVV/2012; Ministry of Education, Youth and Sports CMS 110/2012.

Conflict of interests: None declared.

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Received: October 16, 2011
Accepted: March 28, 2012

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