The stress-related changes in the cerebral blood flow in newborn rats with intracranial hemorrhage: metabolic and endothelial mechanisms

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Abstract. Neonatal brain hemorrhages is a major problem of future generation's health due to the high rate of cognitive disability of newborns after vascular catastrophes in the brain. Despite the public health impact of neonatal brain hemorrhages, the mechanisms underlying in these pathological processes remain unknown. Here, using a model of sound-stress-induced brain hemorrhages (per diapedesis, no per rhexis) in newborn rats and optical methods, we found that brain hemorrhages in newborn rats are accompanied by the increase in perfusion of brain tissues, which closely associated with reducing of cerebral oxygenation and increasing of nitric oxide production in both the brain tissues and blood. We assume that nitric oxide contributes the dilation of cerebral vessels during hypoxia and the increasing of cerebral blood flow in newborn rats with brain hemorrhages. Hypoxic-hyperperfusion during stress-related hemorrhages in newborn animals can be an important protective mechanism against anoxia and critical changes in cerebral hemodynamics.

Keywords: speckle laser contrast imaging, oxygen saturation, stress-related intracranial hemorrhages, hypoxia, nitric oxide.

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1 Introduction

Neonatal brain hemorrhages (BH) are among the top ten causes of development of significant cognitive disability (42%-85% of those who survive) in newborns [1-5]. It was believed that BH is typical only for pre-term newborns and is uncommon among term neonates because the incidence of BH in them is difficult to diagnose due to absence of clear symptoms [6]. However, two magnetic resonance imaging studies of asymptomatic term newborns found 8% (0.2T, 110 neonates) and 26% (3T, 88 neonates) incidence of BH after vaginal delivery [7, 8]. These results show that BH in term newborns is much more frequent that earlier assumed.

The reasons and mechanisms underlying BH are difficult to determine. Potential risk factors for BH are still poorly studied. Some studies report an association between assisted deliveries and BH in neonates, but those results are not consistent [1, 7, 8]. Such risk factors as hypertension, smoking, diabetes, atherosclerosis are not suitable for neonates. It is assumed that stress as the main process of adaptation to the environment is a crucial risk factor for BH in neonates, but the mechanism of stress-related BH is unclear [9, 10].

In this experimental study on three-day-old rat pups, we aimed to determine the mechanisms underlying stress-induced BH. With this aim, we focused on different levels of the cascade of stress responses: systemic (monitoring of cerebral blood flow - CBF), metabolic (assessment of oxygen saturation of the brain tissues) and molecular (evaluation of nitric oxide content in the brain and blood). Our choice of experimental tasks is caused by main actual questions related to the stress-induced cerebrovascular catastrophes. Many studies showed that abnormalities of the CBF, including fluctuations in the CBF, provide a considerable contribution to BH in newborns [11]. Nitric oxide is a main factor of vascular stress-reactivity and resistance to traumatic stress [12-14]. In the central nervous system, NO participates in the regulation of CBF and in the cerebrovascular responses to metabolic activity and to hypercapnia [15, 16]. But, the exact role of these processes in neonatal BH, especially, in aspect of stress accompanying the birth is not established yet and needs to be thoroughly studied.

2 Materials and Methods

Experiments were performed out in newborn mongrel rats 3 days old. All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" [17]. 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).

To induce hemorrhagic stroke, the following protocol of sound stress's impact was used (120 dB, 17 Hz): 10 sec – sound, then 60 sec – pause. This cycle repeated during 2 h [18-20].

For measurement of cerebral circulation, the anesthetized rats (isoflurane – inhalant anesthetic) with fixed head and scalp incision (the dura was left intact) were immobilized. Anesthetic depth was assessed by periodically monitoring the rear foot reflex.

To assess the stress-related changes in cerebral hemodynamics, we studied the changes in relative cerebral blood flow (rCBF or perfusion) using a home-made system for laser speckle contrast imaging (LSCI). The raw speckle images were recorded under the following conditions: light source – HeNe laser with the wavelength 632.8 nm; image sensor – CMOS camera Basler acA2500-14gm; imaging lens – Computer M16140-MP2 16 mm at F-number equal to 6, that corresponds to speckle/pixel size ratio of around 2; exposure time – 20 ms. The speckle images were recorded for 3 min at an average frame rate of 40 frames per second. Spatial speckle contrast was calculated as, $K = \sigma/\langle I \rangle$, where σ is the standard deviation of intensity fluctuations and $\langle I \rangle$ is the mean intensity within a 5x5 sliding window. 50 consecutive frames were averaged into one speckle contrast image.

The measurement of cerebral circulation was performed through the fontanel of newborn rats with focus on:

1) the superior sagittal sinus, which is one of four major sinuses collecting blood from the small veins of the brain;

2) the small, optically unresolvable vessels surrounding the sagittal sinus. Information about macro-

(the sagittal sinus) and microcirculation (capillaries, arterioles, venules) was extracted simultaneously.

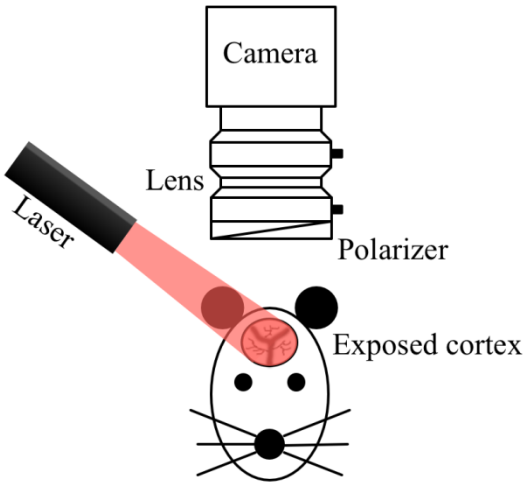

Fig. 1 LSCI system.

Acquisition information from resulting images about flow indexes and vessel's differentiation was performed by histogram analysis of ROI (region of interest). Detailed information about this algorithm and calculation of speckle contrast from raw images itself was explained in our early published article [21] but briefly explanation is following: it was shown several times [22, 23] that conventional LSCI system can sense integral erythrocytes motions even in optically unresolvable vessels. Therefore, if we overlay large enough ROI on speckle image (ROI should contain vessels of different caliber and even unresolvable ones) in resulting histogram we can observe clear peaks. Those peaks are related to different vessels with various diameters. By analysis of this histogram and matching each peaks to a specific collection of vessels with particular diameter we can differentiate venous and microcirculatory components in real time during experiments. In this study rCBF was set to reciprocal of speckle contrast.

The changes in oxygen saturation are often associated with the brain injures and are an important for evaluation of severity of these injures [24]. To measure oxygen saturation level (SpO_2) in the brain tissue we used pulse oximeter model CMS60D (Contec Medical Systems Co., Ltd., Qinhuangdao, China). The system is characterized with a small volume, easy-to-use operation and portability, which make it very convenient for the detection of blood hemoglobin oxygen saturation levels of small laboratory animals. Optical sensor is based on dual wavelengths pulse oximetry approach, using 660 nm and 880 nm for the SpO_2 detection. Display screen of the system could directly show the measured values of the pulse oxygen saturation and pulse rate. Using USB standard for data transmission the measurements could be also visualized on a personal computer using software SpO_2 Assistant version 2.7.0. (Contec Medical Systems Co., Ltd., Qinhuangdao, China). The software allows as well to store the data in ASCII format for a further analysis.

The content of NO (*in vitro*) in serum and cerebral tissues (cerebral cortex, cerebellum, hippocampus, brain axis) was determined by the presence of nitrite. The rats were decapitated, the blood samples were collected, the brain was carefully removed and homogenized. Staining was performed using the Griess reagent-Ilosvaya. The color intensity is measured with a spectrophotometer (SF-2000 Bio, Saint Petersburg, Russia, 2002) at fixed wavelength of 538 nm.

2.1 The protocol of experiments

The basal values of SpO_2 and rCBF were performed in each healthy newborn rats (n=24). Afterward, each rat underwent to sound stress during 2h. Next day after stress when brain hemorrhages developed, we performed the recording of rCBF and SpO_2 in the same newborn rats (n=24). Thus, animals were divided into two groups:

1) intact, unstressed newborn rats (the control group);

2) stressed rats 24 h after stress-off.

The measurement of NO content in the brain tissues and in blood was carried out on other newborn rats: the control group including healthy newborn rats (n=24) and the stressed group (n=24) including newborn rats 24h after stress with brain hemorrhages.

The results were reported as mean ± standard error of the mean (SEM). 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.

3 Results

3.1 Stress-induced changes in the cerebral blood flow in newborn rats with brain hemorrhages

At the first step of our work, we analyzed the stress-induced changes in perfusion and capacitive sectors of cerebral circulation associated with BH in newborn rats using LSCI system.

The sound stress induced the development of small BH (average size 0.005 mm^2) in the cortex and subcortical tissues per diapedesis (no per rhexis), i.e. separated red blood cells squeezed out of the functionally damaged venous vessel walls. Figure 2 (in the right) demonstrates a typical example of diapedesis BH in the subcortical tissues in newborn rat. All rats survived after sound stress, there were no dead newborn rats. Note, that human newborns with BH demonstrate

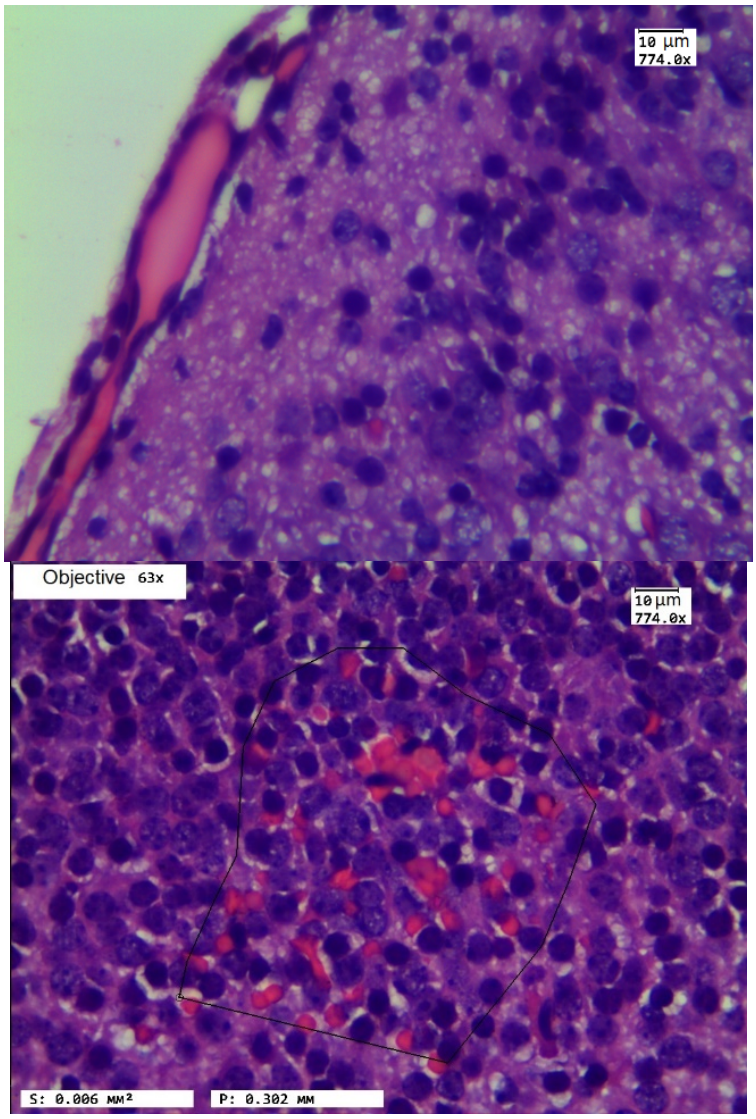

Fig. 2 The typical example of diapedesis (no per rhexis) brain hemorrhage (in the right) in newborn rat (the subcortical area). The normal subcortical tissue of newborn rat from the control group is shown in the left. Hematoxylin & Eosin staining. Bars represent 10 µm (774.0X).

high survives after such brain injures but with long lasting cognitive disorders [1-5]. The one of possible explanation of this fact is the location of brain hemorrhages in neonates who have usually vascular catastrophes in the cortex, i.e. not in the deep important brain centers controlling vital functions such as heartbeats, breathing and etc. [3,6]. Therefore, the consequences of BH in the intellectual zone of the brain might not be recognized for many years [1-5].

LSCI results showed that newborn rats with BH vs. healthy animals demonstrated an increased rCBF in both level of venous and microcirculatory components of cerebral circulation. But, the changes in the sagittal sinus was more pronounced than those in microvessels (Fig. 3). Indeed, after stress exposure rCBF increased in newborn rats by 53±3% ($p<0.05$) in the sagittal sinus and by 29±4% ($p<0.05$) in microvessels. In our previous study we also obtained that stress-reactivity of cerebral veins are greater than vessels of microcirculatory bed [18].

3.2 Stress-induced changes in blood oxygen saturation of the brain tissues in newborn rats with brain hemorrhages

At the second step of this investigation, we studied the metabolic component of cerebral circulation using the assessment of SpO_2 of the brain tissues. The results of this series of experiments showed that the stress-related changes in the CBF in newborn rats was associated with reduced of SpO_2 in the brain. The following SpO_2 levels averaged for all animals were obtained: 98.1±0.3% in the control group and 76.5±3.0% ($p<0.05$) in newborn rats with BH. Thus, the stress-induced pathological changes in the cerebral circulation was associated with reducing of SpO_2 by 22% ($p<0.05$) compared with normal state (Fig. 4).

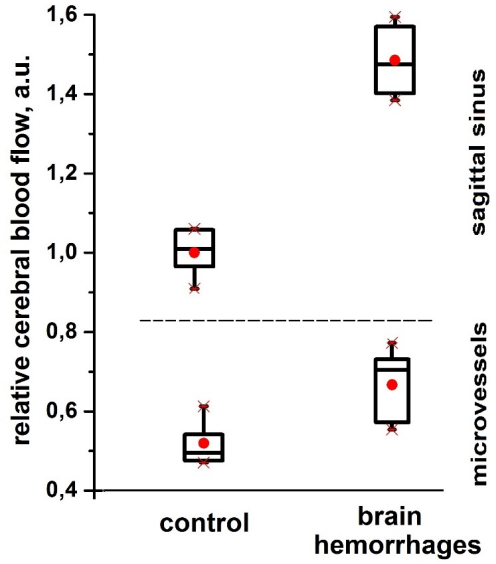

Fig. 3 The relative cerebral blood flow (a.u.) assessed by LSCI in the sagittal sinus and in the microvessels in healthy newborn rats (the control group) and in newborn rat with brain hemorrhages.

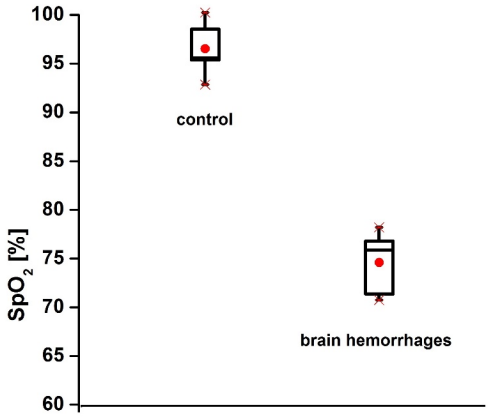

Fig. 4 The SpO_2 (%) in the brain tissues in healthy newborn rats (control group) and newborn rat with brain hemorrhages.

3.3 Stress-induced changes in NO production in the blood and brain tissue in newborn rats with brain hemorrhages

At the third step of this work, we analyzed endothelial component of cerebral circulation using spectrophotometric assessment of NO production in the blood and brain tissues in healthy newborn rats (the control group) and in newborn animals with BH.

The results demonstrated that the increased rCBF and hypoxia, which we obtained in newborn rats with BH in the first and second parts of this work, were associated with the increase in NO content in all investigated zoned of the brain as well as in the blood (Table 1). So, NO level in newborn rats with ICH was higher in the cortex, cerebellum, hippocampus, brain axis, and in the blood compared with healthy animals.

Table 1 Stress-induced changes in NO (μg/ml) production in the blood and brain tissues in healthy (the control group) and with BH newborn rats.

	healthy newborn rats	newborn rats with ICH
cortex	0.23±0.02	0.36±0.03*
cerebellum	0.27±0.03	0.32±0.02*
hippocampus	0.22±0.04	0.31±0.05*
brain axis	0.20±0.03	0.28±0.02*
blood	0.30±0.07	0.48±0.05*

$p<0.05$ vs.: * - healthy newborn rats (control group)

4 Discussion

Here we studied the stress-induced changes in the brain tissues and vessels associated with BH in newborn rats using LSCI assessment of perfusion (microvessels) and capacitive (the sagittal sinus as a main cerebral vein) components of cerebral circulation as well as a measure of metabolic (SpO_2 in the brain tissues) and endothelial (level of NO in the brain tissues and in the blood) factors in the regulation of cerebral blood flow.

Our results show that the stress-induced development of BH in newborn rats is accompanied by the increase in rCBF in both level of microvessels and venous zones of cerebral circulation. These results are consistent with clinical data suggesting that hyperperfusion is one of mechanisms responsible for brain hemorrhages [25-27]. However, separate LSCI analysis of venous and microcirculatory components of cerebral circulation shows that the maturation of changes in the sagittal sinus were more pronounced than in microcirculatory network (53±3% vs. 29±4%, $p<0.05$). This fact is according with our previous histological data, which showed that the venous vessels are more sensitive to harmful effect of stress than microvessels [18]. In clinical studies also have been shown that neonatal stroke is primary venous infarction due to a weakness of the wall of cerebral veins in neonates [28].

We assume that the increase in rCBF in the area of microvessels is the consequence of congestion of blood in the cerebral veins and the decreasing of blood outflow from the brain. In our previous histological data [18-20] we showed that the brain hemorrhages in newborn rats are associated with the increase in size of cerebral veins due to accumulation of extensive blood in them. These changes are associated with formation of perivascular edema, i.e. fluid pathway from the vessels. The relaxation of cerebral veins with perivascular edema are markers of accumulation of extensive blood in venous system and suppression of blood outflow from the brain leading to venous insufficiency [29].

The hypoxia is one of main reasons for critical venous pathological changes associated with brain hemorrhages in newborns [30]. Our results suggest that stress-induced increase in rCBF is accompanied by

reduction of SpO_2 in the brain tissues. Notice, that the hypoxic-hyperperfusion is described also in other studies [31, 32]. A normal physiological response to reduction of oxygen delivery is relaxation of cerebral vessels that activates metabolism in the brain tissues via the increasing of CBF. The particularities of cerebral veins are no muscles and valves in their thin walls, therefore, they have low resistance to critical stretching occurring during blood accumulation in them [33]. The immature brain vessels of newborns have limitation in vasorelaxation capabilities (increase of vessel size). The hypoxia-induced vasorelaxation of cerebral veins causes increasing of cerebral venous pressure [34]. This high pressure can induce easily the rupture of thin walls of immature cerebral veins of newborns [28].

To better understanding of mechanisms underlying relaxation of cerebral vessels during hypoxia in newborn rats with BH, we studied the role of NO in these processes. Our choice of this vascular factor is caused by the fact that NO is a vasodilator, in the central nervous system, NO participates in the regulation of CBF and in the cerebrovascular responses to metabolic activity and to hypercapnia [15, 16]. Thus, NO is complex endothelial factor, which is involved in many levels of regulation of cerebral hemodynamics as well as NO is a key endothelial factor for regulation of vascular tone during stress [12-14].

We show that the stress-induced hypoxic-hyperperfusion in the brain tissues in newborn rats with BH is associated with increasing of NO level in the blood and cerebral tissues. The increase NO concentration was the same in different zones of the brain. But, the serum NO level was higher than in the brain that it is possible to explain by the stress-induced increase in NO synthesis in the vessels of all inner organs. In our previous results, we found that the stress-induced increase in NO serum level is a protective mechanism against the significant pressure effect of stress [12, 13]. Others also demonstrate that the increase of production of major endothelial relaxant such as NO is one of protective mechanism responsible for increasing of tissue perfusion during hypoxia [32]. Taking into account these data, we believe that the elevated level of NO in the brain and blood is the vascular protective response to hypoxia as mechanism of compensation of oxygen deficiency via the increase in perfusion of brain tissues.

5 Conclusion

In summary, our results suggest that stress-induced ICH in newborn rats is accompanied by the increase in perfusion of brain tissues, which closely associated with reducing of SpO_2 in the brain tissues and increasing of NO production in both the brain tissues and blood. We assume that NO as a major endothelial vasorelaxant may contribute dilation of cerebral vessels under hypoxia that explains the increase in rCBF in newborn rats with ICH. Thus, hypoxic-hyperperfusion during stress-related hemorrhages in newborn animals can be an important protective mechanism against anoxia and critical changes in cerebral hemodynamics.

Acknowledgments

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