Experimental Neurology 233 (2012) 481–489

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Cerebral blood flow during reperfusion predicts later brain damage in a mouse and a rat model of neonatal hypoxic–ischemic encephalopathy

Makiko Ohshima [a], Masahiro Tsuji [a,*], Akihiko Taguchi [a], Yukiko Kasahara [a], Tomoaki Ikeda [a,b]

[a] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishiro-dai, Suita, Osaka, 565-8565, Japan
[b] Department of Perinatology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishiro-dai, Suita, Osaka, 565-8565, Japan

ARTICLE INFO

Article history:
Received 12 September 2011
Accepted 11 November 2011
Available online 27 November 2011

Keywords:
Hypoxic–ischemic brain injury
Cerebral blood flow
Reperfusion
Laser speckle flowmetry
Neonatal
Rat
Mouse
Dexamethasone

ABSTRACT

Children with severe neonatal hypoxic–ischemic encephalopathy (HIE) die or develop life-long neurological impairments such as cerebral palsy and mental retardation. Decreased regional cerebral blood flow (CBF) is believed to be the predominant factor that determines the level of tissue injury in the immature brain. However, the spatio-temporal profiles of CBF after neonatal HIE are not well understood. CB17 mouse and Wistar rat pups were exposed to a unilateral hypoxic–ischemic (HI) insult at eight or seven days of age. Laser speckle imaging sequentially measured the cortical surface CBF before the hypoxic exposure and until 24 h after the hypoxic exposure. Seven days after the HI insult, brain damage was morphologically assessed by measuring the hemispheric volumes and by semi-quantitative scoring for neuropathologic injury. The mean CBF on the ipsilateral hemisphere in mice decreased after carotid artery ligation. After the end of hypoxic insult (i.e., the reperfusion phase), the mean CBF level gradually rose and nearly attained its pre-surgery level by 9 h of reperfusion. It then decreased. The degree of reduced CBF during reperfusion was well correlated with the degree of later morphological brain damage. The correlation was the strongest when the CBF was measured in the ischemic core region at 24 h of reperfusion in mice ($R^2 = 0.89$). A similar trend in results was found in rats. These results suggest that the CBF level during reperfusion may be a useful predictive factor for later brain damage in immature mice. This may enable optimizing brain damage for detail analyses.

Introduction

Children with severe neonatal hypoxic–ischemic encephalopathy (HIE) typically die or develop life-long neurological impairments such as cerebral palsy, mental retardation, and epilepsy (van Handel et al., 2007). No therapeutic method is available for perinatal HIE, apart from initiating hypothermia within 6 h after birth. An important issue in neonatal practice is the early detection of pathophysiological factors that are associated with permanent brain damage. Extensive laboratory research with experimental models is being performed to understand the mechanisms of brain injury and to find neuroprotective therapies. The Rice–Vannucci model, which combines permanent unilateral ligation of a carotid artery with exposure to hypoxia for 2 to 3 h in seven-day-old rat pups, has been widely used to study the physiological and therapeutic variables of neonatal hypoxic–ischemic (HI) injury (Johnston et al., 2005; Rice et al., 1981). This model has also been adapted for use in neonatal mice.

Decreased regional cerebral blood flow (CBF) is the predominant factor that determines the topography of tissue injury in the immature rodent brain, although metabolic factors (i.e., intrinsic vulnerability) may influence injury in some brain structures (Ringel et al., 1991; Vannucci et al., 1988). In immature rats with an HI injury, radioactive tracers in coronal sections show a residual columnar perfusion deficit within the cerebral cortex, which corresponds closely to the pathological pattern of injury within this structure. The pathological pattern of injury is characterized by alternating columns of normal and damaged neurons, which are oriented at a right angle to the pial surface. CBF is rarely monitored during and after HI insult in immature rodent models, mainly because of the technical difficulties and invasiveness involved in measuring it. Therefore, little is known about the spatial and temporal extent of CBF response during the reperfusion period after an HI insult.

The autoradiographic techniques for analyzing CBF in small animals entail sacrificing the animals at the time of measurement. A less invasive method, laser Doppler flowmetry (LDF), has been widely used to monitor relative perfusion changes. The LDF technique uses a single-point measurement in which the hemodynamics of an area covering 1 mm^3 to 2 mm^3 can be measured from the tip of a probe (Stern et al., 1977). CBF measured at a small arbitrarily selected area may not accurately reflect the hemodynamics of the cerebral hemisphere, and the

Abbreviations: NHIE, neonatal hypoxic–ischemic encephalopathy; HI, hypoxic–ischemic, hypoxia–ischemia; CBF, cerebral blood flow; LSF, laser speckle flowmetry; LDF, laser Doppler flowmetry; ROI, region of interest; MCA, middle cerebral artery; MRI, magnetic resonance imaging; DWI, Diffusion-weighted MRI; ANOVA, analysis of variance.

* Corresponding author. Fax: +81 6 6835 5496.
E-mail addresses: oshimam@ri.ncvc.go.jp, mtsujimd@ybb.ne.jp (M. Ohshima), mtsuji@ri.ncvc.go.jp (M. Tsuji), taguchi@ri.ncvc.go.jp (A. Taguchi), kasahara@ri.ncvc.go.jp (Y. Kasahara), tikeda@hsp.ncvc.go.jp (T. Ikeda).

0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.11.025

probe-position-specific manner of measurement makes repeated intermittent measurements unreliable. Since LDF generally requires the attachment of a probe onto the skull or the dura, long-term observation would excessively strain the pups. Hence, the monitoring duration with LDF in most cases is short, being less than 1 (Liu et al., 1999; Matsiukevich, et al., 2010; Taniguchi et al., 2007), or 2 h after the end of hypoxic exposure (Fabian et al., 2008; Ioroi et al., 1998). To our knowledge, there is only one report in which the CBF was observed beyond the early perfusion phase in a rodent model of neonatal HIE (Wainwright et al., 2007). Laser Doppler perfusion imaging is a recent development in LDF that extends its power to the two-dimensional measurement of tissue perfusion. However, laser Doppler perfusion imaging cannot be used to dynamically image high frequency blood-flow fluctuations since the temporal separation between the first and last image points within a scan can be several minutes. In addition, its resolution is not very high, ranging from 100 μm/pixel to 1.0 mm/pixel (Forrester et al., 2002; Riyamongkol et al., 2002).

The laser speckle method for imaging vascular structure in a tissue has been available since the 1980s. It has recently been revised to measure CBF, as first described by Dunn et al. (2001). For a duration of several milliseconds to several hours, the speckle imaging method is able to accurately image the cortical blood-flow response over an area ranging from a few millimeters to the whole rodent brain (Dunn et al., 2001). Laser speckle flowmetry (LSF) provides excellent spatial resolution, even through an intact skull. This allows the measurement of CBF changes occurring within the pial vasculature (Ayata et al., 2004). The laser speckle technique primarily measures the velocity of scattering particles (e.g., red blood cells). LSF provides an index of perfusion that has a linear relationship with the absolute CBF value (which is measured by the [^{14}C]iodoamphetamine technique (Ayata et al., 2004) and by the clearance rate of umbelliferone (Strong et al., 2006)). LSF enables the long-term observation of CBF (Fujita et al., 2010). To our knowledge, the use of two-dimensional laser speckle perfusion imaging to observe CBF has never been investigated in immature rodent models of HI insult or stroke.

There is significant inter- and intra-litter variability in the extent of the brain injury in the HI model: a subset of pups suffers no perceivable brain injury, while other pups suffer massive hemispheric infarct (Sheldon et al., 1998). This also holds true in neonatal stroke models (Bonnin et al., 2011; Comi, et al., 2005; Wendland et al., 2008). The variability in animal models resembles the variability seen in human infants with neonatal HIE. In a population-based study, the teenage outcome of children who had been born with moderate neonatal HIE was quite variable: 35% had cerebral palsy or other major neuroimpairments; 46% had cognitive problems without cerebral palsy; and 9% had no problems (Lindström et al., 2006). Hence, it is no wonder that some pups may have no lesion after an HI insult. The variability in animal models, however, makes detailed preclinical analyses difficult to perform. Efforts have been made to offset this hindrance and, in particular, to exclude subjects with no lesion, but there is no widely used method to optimize degree of brain injury. A few laboratories use parameters—such as apparent diffusion coefficient (ADC) obtained by magnetic resonance imaging (MRI) (Derugin et al., 2000; Wendland et al., 2008), or the CBF obtained by color-coded pulsed Doppler ultrasound imaging (Bonnin et al., 2011)—to exclude pups without a lesion at an early stage of brain injury. The objectives of our study are: 1) to show temporal changes in CBF in a mouse model and in a rat model of neonatal HIE and 2) to examine the correlation between CBF during the early stage of HI injury and later morphological brain damage.

Materials and methods

Hypoxia–ischemia procedure

All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center. Eight-day-old (postnatal day 8, [P8]) male and female CB17 mouse pups (CLEA Japan Inc., Tokyo, Japan) and seven-day-old (P7) male and female Wistar rat pups (Japan SLC Inc., Hamamatsu, Japan) were prepared for surgery. Under isoflurane anesthesia (4.0% for induction and 1.5% to 2.0% for maintenance), the left carotid artery was permanently occluded in the mouse and rat pups. After a one- to two-hour recovery period, the mouse pups were subjected to hypoxia (8% oxygen and 92% nitrogen, at 33.0 °C) for 30 min and the rat pups were subjected to hypoxia for 120 min. After a 60-min recovery period in a temperature-controlled incubator, the pups were returned to their dams and kept in a standard environment.

Laser speckle blood-flow imaging

In 23 mice and 33 rats, the cortical surface CBF was sequentially measured by a laser speckle flowmetry (LSF) imaging system (Omegazone, Omegawave Inc., Tokyo, Japan) at several time points: pre-surgery; pre-hypoxia (which is post-surgery); and 0 h, 1 h, 2.5 h, 6 h, 9 h, and 24 h after the end of hypoxia (i.e. after the start of reperfusion). The theory and technique for LSF have been described in detail elsewhere (Dunn et al., 2001; Forrester et al., 2002). In brief, the animals were placed in a prone position and spontaneously breathed under isoflurane anesthesia. The animal's skull was exposed by a midline scalp incision and the skull surface was diffusely illuminated by a 780 nm laser light. The penetration depth of the laser is approximately 500 μm. The scattered light was filtered and detected by a charge coupled device (CCD) camera positioned above the animal's head. Raw speckle images were used to compute the speckle contrast, which is a measure of speckle visibility that is related to the number and velocity of moving particles (in this case, CBF). Color-coded blood-flow images were obtained in the high-resolution mode (638 pixels × 480 pixels; 1 image/s). Five consecutive raw speckle images were acquired at 1 Hz, and then averaged. For analytical accuracy in repositioning of the animal's head and regions of interest (ROIs) between imagings, we set the size and the position of an ROI, based on a line drawn from the bregma to the lambda (Fig. 1A). We measured the CBF in three ROIs: the Core (the ischemic core region of the middle cerebral artery (MCA) territory); the Penumbra (the penumbra region of the MCA territory by the sagittal suture); and the MCA region (the broader region covering most of the MCA territory, including the Core and the Penumbra) (Fig. 1A). The same grid was used to set the three matching regions on the contralateral side. The total measuring procedure took approximately 3 min per pup.

Quantitative histological analysis

Seven days after the HI insult, the animals were deeply anesthetized with an overdose of pentobarbital and intracardially perfusion-fixed with 4% paraformaldehyde. After the perfusion, an animal's brain was removed and coronally sectioned in slices 2-mm thick by using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The area (in mm^2) of the contralateral and ipsilateral hemispheres in each brain section was measured, using NIH Image software (ImageJ, 1.43r, NIH, Bethesda, USA). The hemispheric volume of the brain of each pup was estimated by summing the hemispheric area of the brain slices and multiplying the sum by the section interval thickness. The injury was evaluated by using hematoxylin–eosin-stained sections from four brain regions: the cortex, striatum, hippocampus, and thalamus. We used the system we previously developed for evaluating neuropathologic injury in the present study (Tsuji et al., 2004). Neuropathologic injury in the cerebral cortex was scored on a scale ranging from 0 to 4 points (0, no injury; 4, extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored on a scale ranging from 0 to 6 points. The total score (ranging from 0 to 22 points) was the sum of these ratings. The