Rodent brain slice model for the study of white matter injury

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Objective: Cerebral white matter (WM) injury is common after cardiac surgery in neonates and young infants who have brain immaturity and genetic abnormalities. To understand better the mechanisms associated with WM injury, we tested the adequacy of a novel ex vivo brain slice model, with a particular focus on how the maturational stage modulates the injury.

Methods: To replicate conditions of cardiopulmonary bypass, we transferred living brain slices to a closed chamber perfused by artificial cerebrospinal fluid under controlled temperature and oxygenation. Oxygen-glucose deprivation (OGD) simulated circulatory arrest. The effects of maturation were investigated in 7- and 21-day-old mice (P7, P21) that are equivalent in maturation stage to the human fetus and young adult.

Results: There were no morphologic changes in axons after 60 minutes of OGD at 15°C in both P7 WM and P21 WM. Higher temperature and longer duration of OGD were associated with significantly greater WM axonal damage, suggesting that the model replicates the injury seen after hypothermic circulatory arrest. The axonal damage at P7 was significantly less than at P21, demonstrating that immature axons are more resistant than mature axons. Conversely, a significant increase in caspase3^{+} oligodendrocytes in P7 mice was identified relative to P21, indicating that oligodendrocytes in immature WM are more vulnerable than oligodendrocytes in mature WM.

Conclusions: Neuroprotective strategies for immature WM may need to focus on reducing oligodendrocyte injury. The brain slice model will be helpful in understanding the effects of cardiac surgery on the immature brain and the brain with genetic abnormalities. (J Thorac Cardiovasc Surg 2013;146:1526-33)

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Both prospective clinical trials and retrospective clinical studies have documented significant neurodevelopmental deficits in children with congenital heart disease (CHD).[1-3] Recent studies using magnetic resonance imaging have identified evidence of white matter (WM) injury in neonates and young infants with CHD.[4-6] Major clinical correlates of WM injury are gross and fine motor deficits, which are the most common neurologic deficits seen in children after cardiac surgery.[7-9] In addition, recent advances in the field of neuroscience illuminate an important role of WM structure in specific cognitive functions, including reading, verbal function, executive decision making, working memory, and learning complex skill.[10] Interestingly, impairments of these functions are largely observed in school-aged children and adolescents who have had cardiac surgery for CHD.[2,8,11] Therefore, understanding the cellular and molecular events that result in such WM injury is of crucial importance to develop targeted therapies and conditions that will minimize the risk of neurologic deficits in patients with CHD.[12]

Cellular and molecular processes underlying WM injury and its repair have been extensively explored in rodent animal models based on the tremendous power of transgenic and gene knockout technologies.[13] This approach has assisted in establishing novel therapeutic strategies for WM disorders such as multiple sclerosis.[14] We have recently introduced cutting-edge neuroscience techniques to study WM injury in the porcine model[15]; however, transgenic and gene knockout technologies are still extremely limited in large animals. Thus, for further investigation of WM injury and the development of novel treatment approaches, it is imperative to explore new animal models that replicate pathologic conditions to which the brain is exposed under CHD and subsequent cardiac surgery.

Clinical studies have reported a significant high incidence (25%-55%) of newly developed WM injury after cardiac surgery.[4-6] The major brain insults during surgery include

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Abbreviations and Acronyms

aCSF = artificial cerebrospinal fluid
CHD = congenital heart disease
CNP = 2′,3′-cyclic nucleotide 3′phosphodiesterase
CPB = cardiopulmonary bypass
DHCA = deep hypothermic circulatory arrest
OGD = oxygen-glucose deprivation
PBS = phosphate-buffered saline
WM = white matter

cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA).[8,16] DHCA is a unique and specific pathologic condition in patients undergoing cardiac surgery, which causes ischemia-reperfusion/reoxygenation under hypothermia. To reproduce conditions of CPB and DHCA, we developed a unique brain slice model in which living brain slices are transferred to a closed circulation system perfused by artificial cerebrospinal fluid (aCSF) under controlled temperature, pH, and oxygenation. In the present study, we examined the adequacy of our brain slice model for the investigation of WM injury associated with DHCA. We assessed WM injury owing to hypothermic ischemia-reperfusion/reoxygenation using 2 transgenic mice strains with a focus on axons and oligodendrocytes, which are major cellular components in the WM.[13,14] Inasmuch as recent clinical studies identified brain maturation as an important factor in WM injury after cardiac surgery,[5] we studied how maturation stage modulates the damage in WM axons and oligodendrocytes.

METHODS

Animals

Two lines of transgenic mice were studied. In thy1-yellow fluorescent protein-16 (C57BL/6) mice, yellow fluorescent proteins are selectively expressed in neuronal bodies and axons. In 2′,3′-cyclic nucleotide 3′phosphodiesterase (CNP) mice, human enhanced green fluorescent protein is overexpressed in the oligodendrocyte lineage under the CNP promoter.

Brain maturation in newborns with CHD is delayed approximately 1 month.[17,18] Therefore, the effects of brain maturation on WM injury were investigated in both 7- and 21-day-old mice (P7, P21) that have WM maturation equivalent to the human fetus (35-36 weeks of gestation) and to a 10-year-old child, respectively.[19] We performed all experiments in compliance with the "Guide to the Care and Use of Laboratory Animals" published by the National Institutes of Health. The study was approved by the Animal Care and Use Committee of the Children's National Medical Center.

Brain Slice Preparation

The brains were collected from 2 transgenic mice strains at P7 and P21 and were dissected out into ice-cold 95% oxygenated modified aCSF slicing solution composed of the following (in millimoles per liter): 87 NaCl, 2.5 KCl, 3 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, and 75 sucrose, pH 7.4. Coronal sections, 400 μm, were cut using a vibratome (Leica 1200 VT; Leica Microsystme Inc, Buffalo Grove, Ill). Slices were allowed to recover at 22°C in the slicing solution for 1 hour. Perfusate temperature was then gradually increased up to 36°C over 2 hours as is the practice for electrophysiologic studies using murine brain slices.[20] Only slices containing corpus callosum, which is a major WM structure in the mouse brain, were included in the experiments (2-3 per animal). The slices from 3 littermates (6-9 per experiment) were transferred to a customized tissue chamber (CellMicro, Norfork, Va; Figure 1, *A* and *B*).

In the closed chamber system, brain slices were perfused (2 mL/min) with aCSF composed of the following (in millimoles per liter): 126 NaCl, 3.5 KCl, 1.3 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose, pH 7.4; saturated with 95% oxygen/5% carbon dioxide. A heating/cooling device (CellMicro, Norfork, Va) was connected with the system to control the temperature of the aCSF. The bottom of the chamber itself also contained the temperature-control device (CellMicro). This bipolar temperature controller allows precise temperature control in slices being studied. Brain slice preparation and perfusion methods were modified from previous reports.[20,21]

Oxygen-Glucose Deprivation

To replicate DHCA, we exposed slices to oxygen-glucose deprivation (OGD) (Figure 1, *A*). OGD was achieved by changing the perfusion solution from aCSF saturated with 95% oxygen/5% carbon dioxide to glucose-free aCSF (supplemented with 10 mmol/L sucrose to maintain osmolarity) saturated with 95% nitrogen/5% carbon dioxide. The slice was reperfused with glucose-containing aCSF saturated with 95% oxygen/5% carbon dioxide after the period of OGD, providing ischemia-reperfusion/reoxygenation to the brain slices. This technique has been widely used to investigate ischemia-induced brain injury in culture systems.[21,22] In the present study, the OGD was performed under 3 hypothermic conditions using the temperature-controlled circulation system described above. The contents of oxygenated aCSF and aCSF under OGD are shown in Table E1.

Perfusion Protocol

Each experiment was assigned to 1 of 3 groups with different temperatures of 60 minutes of OGD (15°C, 25°C, and 36oC OGD). After the initial recovery period after the slice procedure, brain slices were cooled to a temperature of 15°C, 25°C, or 36°C for 30 minutes according to the protocol. The OGD was then performed for 60 minutes under 3 temperature settings. After the OGD, slices were reperfused with glucose containing aCSF saturated with 95% oxygen/5% carbon dioxide in 5 minutes, followed by a 30-minute rewarming period (Figure 1, *C*). This replicates DHCA-induced hypothermic ischemia-reperfusion/reoxygenation. Damage to WM axons and oligodendrocytes in brain slices was assessed at 0 to 20 hours after rewarming. Control slices, which were perfused at 36°C with aCSF saturated with 95% oxygen/5% carbon dioxide for the same duration (no-OGD), were used for comparison with the OGD groups.

Tissue Preparation and Immunohistochemistry

At the conclusion of each experiment, slices were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for 1 hour at room temperature. Each slice was then cryoprotected for 48 hours in 30% sucrose in PBS. All slices were stored at −80°C until further processing. For assessment of WM axonal and oligodendrocyte injury, 20-μm sections were developed from each 400-μm brain slice using a cryotome. Sections from the outer 100 μm of each slice were excluded from the assessment to avoid tissues that may have been damaged during slice preparation. For immunohistochemistry, 20-μm sections were incubated for 1 hour at room temperature with blocking solution (10% normal goat serum, 1% bovine serum albumin, and 0.3% Tween 20 in PBS, pH 7.4) and then incubated at 4°C overnight with primary antibody and carrier solution (1% normal goat serum, 1% bovine serum albumin, and 0.3% Tween 20 in PBS, pH 7.4). Sections were washed with PBS and then incubated for 1 hour at room temperature with secondary antibody and carrier solution.[15,20] An antibody to cleaved caspase-3 was used to identify apoptosis in the WM.[15]