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Rodent neonatal germinal matrix hemorrhage mimics the human brain injury, neurological consequences, and post-hemorrhagic hydrocephalus

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ABSTRACT

Germinal matrix hemorrhage (GMH) is the most common neurological disease of premature newborns. GMH causes neurological sequelae such as cerebral palsy, post-hemorrhagic hydrocephalus, and mental retardation. Despite this, there is no standardized animal model of spontaneous GMH using newborn rats to depict the condition. We asked whether stereotactic injection of collagenase type VII (0.3 U) into the ganglionic eminence of neonatal rats would reproduce the acute brain injury, gliosis, hydrocephalus, periventricular leukomalacia, and attendant neurological consequences found in humans. To test this hypothesis, we used our neonatal rat model of collagenase-induced GMH in P7 pups, and found that the levels of free-radical adducts (nitrotyrosine and 4-hyroxynonenal), proliferation (mammalian target of rapamycin), inflammation (COX-2), blood components (hemoglobin and thrombin), and gliosis (vitronectin and GFAP) were higher in the forebrain of GMH pups, than in controls. Neurobehavioral testing showed that pups with GMH had developmental delay, and the juvenile animals had significant cognitive and motor disability, suggesting clinical relevance of the model. There was also evidence of white-matter reduction, ventricular dilation, and brain atrophy in the GMH animals. This study highlights an instructive animal model of the neurological consequences after germinal matrix hemorrhage, with evidence of brain injuries that can be used to evaluate strategies in the prevention and treatment of post-hemorrhagic complications.

Introduction

Germinal matrix hemorrhage (GMH) is the most common neurological disorder of newborns, and is defined as the rupture of immature blood vessels within the subependymal brain tissue of the premature infant (Ballabh, 2010; Vermont-Oxford, 1993). This occurs approximately 3.5 times per 1000 live births (Heron et al., 2010) and is an increasing socio-economic problem since both the preterm birth rates, and neonatal survival, have considerably increased over the past two decades (Shennan and Bewley, 2006). The major neurological sequelae following intraventricular extension of GMH are: cerebral palsy, post-hemorrhagic hydrocephalus (PHH) and debilitating cognitive deficits (e.g. mental retardation and academic difficulties) (Ballabh et al., 2004; Bassan et al., 2007).

Since GMH has been largely unpreventable (Bassan, 2009; Roland and Hill, 2003), and because clinical treatments are mostly inadequate (supportive) (Kenet et al., 2011), it is clinically important to develop and test novel therapeutic strategies to mitigate these devastating neurological consequences. Thus, it is necessary to characterize standardized animal models to study the spectrum of brain injuries and neurobehavioral deficits following GMH, in order that neuroprotective and preventative modalities can be adequately developed and tested for this vulnerable patient population (Ballabh et al., 2007; Chua et al., 2009; Zia et al., 2009).

GMH has been modeled using several animal species, including the rabbit, dog, sheep, rat, mouse, and pig, either by direct (needle) injection of blood into the ventricle, or by changing hemodynamic (systemic) properties, including blood pressure, circulating blood volume, serum glycerol, carbon-dioxide, osmolarity, or oxygenation levels (Balasubramaniam and Del Bigio, 2006; Georgiadis et al., 2008; Goddard et al., 1980). However, these animal models do not adequately resemble the premature neonates having GMH with respect to the etiology, neuropathology, and clinical outcomes of the disease (Aquilina et al., 2007, 2011; Balasubramaniam et al., 2006; Cherian et al., 2003, 2004). Although needle insertion into the brain has inherent disadvantages of producing direct trauma to surrounding tissues, the infusion of blood has little relation to a spontaneous bleed, and the modification of hemodynamic factors leading to hypoxia, hypertension, hypercarbic, hyperosmotic, or hypervolemic states will

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confound the GMH-induced brain pathology. Another limitation of existing models is the cost and the special expertise required to care for and rear prematurely delivered large mammals used to model GMH. Rodent models of brain injury are relatively easy to use, maintain, inexpensive to reproduce, and their neurobehavior development has been well documented (Altman and Sudarshan, 1975). However, there is a lack of rodent model bearing neurological consequences of GMH that naturally mimics the preterm survivors of GMH.

We thus chose to administer collagenase (sterile intracerebral hemorrhage inducing agent (Rosenberg et al., 1990)) for induction of GMH, because it will cause a standardized, stereotaxically-controlled, and spontaneous rupture of the ganglionic eminence into the lateral ventricles (Lekic et al., 2011a, 2011b). We then asked whether the induction of GMH would be attended by significant regional brain swelling (edema), delays of neurodevelopment, increased fibrogenesis and gliosis, altered body or brain growth, and cognitive or motor deficits. Finally, we evaluated the juvenile brains to demonstrate the atrophy, ventriculomegaly, and clinical relevance of the model.

Materials and methods

Animal groups and general operative procedures

This study was conducted in accordance with the National Institutes of Health guidelines for the treatment of animals, and was approved by the Institutional Animal Care and Use Committee of Loma Linda University. Timed pregnant Sprague–Dawley rats were purchased from Harlan Laboratories, Indianapolis, IN. One hundred fifty three P7 rat pups were then randomly divided into the following groups: sham-operated (n = 41), needle-control (n = 18), collagenase-0.1 units (n = 10), and collagenase-0.3 units (n = 84). Pups of both genders were subjected to the operative procedure, using aseptic technique, they were gently anesthetized with 3% isoflurane (in mixed air and oxygen), while placed prone onto a stereotaxic frame. For GMH induction, betadine first sterilized the surgical scalp area, which was then incised in the longitudinal plane to expose the skull and reveal the bregma. The following stereotactic coordinates were precisely measured from bregma: 1.8 mm (rostral), 1.5 mm (lateral), and 2.8 mm (depth) from the dura. A burr hole (1 mm) was drilled, into which a 27 gauge needle was inserted at a rate of 1 mm/min. A microinfusion pump (Harvard Apparatus, Holliston, MA) infused 0.3 units of clostridial collagenase VII-S (Sigma, St Louis, MO) through the Hamilton syringe. The needle remained in place for an additional 10 min after injection to prevent "back-leakage". After needle removal, the burr hole was sealed with bone wax, the incision suture closed, and the animals were allowed to recover on a 37 °C heated blanket. The entire surgery took on average 20 min. Upon recovering from anesthesia, the animals were returned to their dams. Needle-controls consisted of needle insertion alone without collagenase infusion.

Experiment 1: mechanisms of Brain Injury following GMH

Animal perfusion and tissue extraction

The animals were fatally anesthetized with isoflurane (≥5%) followed by cardiovascular perfusion with ice-cold PBS for hemoglobin assay, thrombin level, and immunoblot analyses. The forebrains were dissected and snap-frozen with liquid-nitrogen, then stored in −80 °C freezer, before protein extractions, or spectrophotometric quantification.

Brain water content

The percentage of brain edema was measured using the wet-weight/dry-weight method 24 h after GMH induction (Tang et al., 2005). Quickly following sacrifice, the brains were removed and divided. Tissue weights were determined before and after drying for 24 h in a 100 °C oven, using an analytical microbalance (model AE 100; Mettler Instrument Co., Columbus, OH) that is capable of measuring within 1.0 μg of precision. Brain edema was then finally calculated as a percentage: (wet weight − dry weight)/wet weight × 100.

Hematoma size

The hemorrhagic injury size was quantified 24 h after GMH induction using computer-assisted outlining of brain slices. Neonatal rats were euthanized under deep (≥5%) isoflurane anesthesia. The brains were removed and cut into slices using a 1-mm rat brain matrix. Under standardized conditions, images of the brain slices were taken with a digital camera, and then converted into a binary image for the area delineation analysis using Image J software (National Institutes of Health, Bethesda, MD). Further details have published elsewhere (Chang et al., 2011; Foerch et al., 2008).

Composite neuroscore

The 24-hour neurological evaluation consisted of a sensorimotor value represented by the combined averages from negative geotropism, righting reflex, and grip traction test as described (Balasubramaniam et al., 2006; Cherian et al., 2003; Thullier et al., 1997). The values are expressed as percent of sham, and further procedural details are provided below (in Experiment 2).

Western blotting

For the protein immunoblot (Lekic et al., 2011a, 2011b), the concentration was first determined using the DC protein assay (Bio-Rad, Hercules, CA). Samples were subjected to SDS-PAGE on 4–20% gels and then transferred to nitrocellulose membrane for 100 min at 100 V (Bio-Rad). The blotting membranes were incubated for 1 h with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. These were then incubated overnight with the following primary antibodies: anti-Vitronectin (1:25 000; Abcam, Cambridge, MA), anti-GFAP (1:20 000; Abcam, Cambridge, MA), anti-COX2 (1:200; Cayman Chemical, Ann Arbor, MI), or anti-phospho-MTOR (1:1000; Cell Signaling Technology, Danvers, MA). The membranes were then incubated with secondary antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) and then processed with the ECL plus kit (GE Healthcare and Life Science, Piscataway, NJ). For the internal control, the same membrane was probed with an antibody against β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) after being stripped. The relative density of resultant protein immunoblot images were finally semi-quantitatively analyzed using Image J software (4.0, Media Cybernetics, Silver Spring, MD) as described (Tang et al., 2004).

Hemoglobin assay

The spectrophotometric measurement of hemorrhagic volume was performed using well-established protocols (Choudhri et al., 1997; Tang et al., 2004). Extracted forebrain tissue was placed in glass test tubes with 3 mL of PBS, and then homogenized for 60 s (Tissue Miser Homogenizer; Fisher Scientific, Pittsburgh, PA). After ultrasonication for 1 minute lysed erythrocyte membranes; the products were then centrifuged for 30 min, and Drabkin's reagent was added (Sigma-Aldrich) into aliquots of supernatant, which reacted for 15 min. Absorbance, using a spectrophotometer (540 nm; Genesis 10uv; Thermo Fisher Scientific, Waltham, MA), was calculated into a hemorrhagic volume (μL) on the basis of a standard curve as routinely performed (Lekic et al., 2011a, 2011b).