Neuroscience Letters 684 (2018) 29–34

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research article

Repeated mild shaking of neonates induces transient cerebral microhemorrhages and anxiety-related behavior in adult rats

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ARTICLE INFO

Keywords:
Shaken baby syndrome
Animal model
Microhemorrhage
Susceptibility weighted imaging
Iron histochemistry
Anxiety

ABSTRACT

Growing evidence suggests that neonatal cerebral microhemorrhages (MHs) are implicated in neuropsychiatric diseases in adults. Although animal studies have identified the progression of the underlying mechanisms of MHs, few studies have investigated the histopathology and behavioral outcomes. In this study, we created an experimental rat model of MHs using a new experimental device for repeated mild shaking brain injury (SBI) in the neonatal period and examined temporal changes in MHs using susceptibility weighted imaging (SWI) and iron histochemistry. SWI demonstrated transient MHs in the gray matter of the cerebral cortex and hippocampus in injured rats. Iron histochemical staining demonstrated leakage of iron and iron-positive cells surrounding MHs. This staining pattern lasted for a long time and continued after disappearance of hemorrhagic signals on SWI. These data suggested the presence of iron-associated gray matter injury after MHs. In the open field test, these injured rats showed anxiety-related behavior as adults. This model may be useful for exploring the underlying mechanisms of changes that occur after MHs and the behavioral outcomes of repeated mild SBI in early development.

1. Introduction

Abusive head trauma in childhood, known as “shaken baby syndrome” (SBS), is considered to increase the risk for psychiatric disorders in adolescents and adults [1,2]. In SBS, characteristic pathological features of violent head shaking are various combinations of subdural, subarachnoid, and intracerebral hemorrhages and skull fractures [3]. Hemorrhages of various sizes can occur in SBS brains, and over 30% of SBS victims die due to such hemorrhages [3]. However, small hemorrhages and/or those without severe neurological signs are difficult to accurately diagnose and so far have received less attention. To investigate the pathological changes in the developing brain following shaking brain injury (SBI) and its relationship to changes in behavior, several animal models for SBS have been developed [4,5]. In these models, rodents are advantageous for studies of molecular and histopathological mechanisms following shaking insults [6].

Improved and advanced magnetic resonance imaging techniques such as susceptibility weighted imaging (SWI) can detect cerebral microhemorrhages (MHs) [7]. In SWI studies in humans, MHs are an imaging biomarker of “small vessel disease” in elderly people, whereas the presence of hemosiderin (a marker of earlier sustained hemorrhages) in the brain has been used as a prognostic indicator in preterm infants [8]. Although MHs have been demonstrated with SWI after traumatic brain injury (TBI) in model animals [9], no study has reported MHs after SBS and/or SBI in models. In the present study, we designed and built a new apparatus for repeated mild SBI to produce MHs in neonatal brains. Using SWI and histochemical staining, temporal changes in MHs were studied following neonatal repeated SBI.

Hemorrhages in the neonatal brain cause more severe injuries than in the adult brain [10]. Several mechanisms have been proposed for hemorrhage-induced tissue injury. Iron is a central player in such mechanisms. When red blood cells (RBCs) degenerate, hemoglobin liberates oxidized heme and further degrades into iron, bilirubin, and carbon monoxide. Iron can lead to formation of reactive oxygen species, which can promote cytotoxicity [11]. To identify the changes in iron distribution after SBI, we further examined changes in iron in the SBI model using modified Perls staining [12]. Finally, the effects of SBI on long-term emotional outcomes were examined in the novel open field test.

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https://doi.org/10.1016/j.neulet.2018.06.059
Received 12 May 2018; Received in revised form 19 June 2018; Accepted 30 June 2018
Available online 02 July 2018

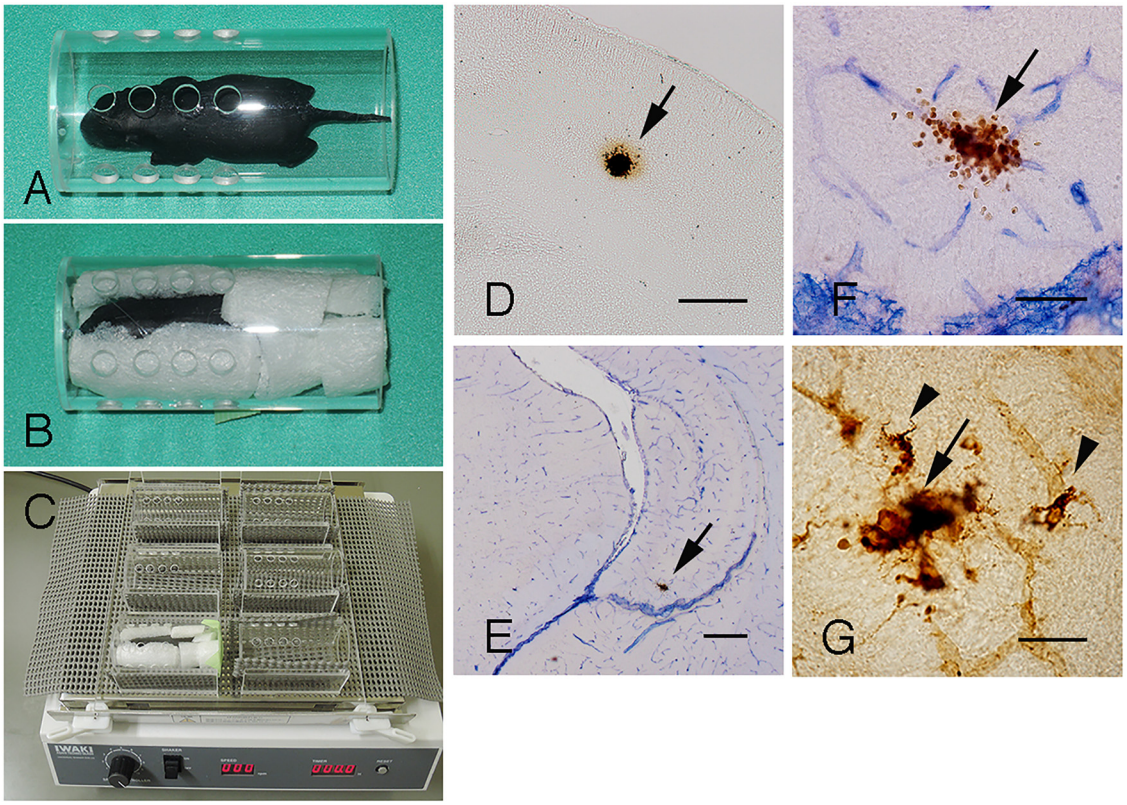

Fig. 1. Shaking apparatus (A–C). (A) The whole body mold for P3 rats was made with epoxy resin. The surface of the mold was painted black. The mold was inserted into the holding chamber, which was made with transparent polyvinyl chloride. (B) The pup is fixed in the mold with cushioning materials made of plant starch and polyvinyl alcohol, and the mold was placed in the chamber. (C) The shaking apparatus is composed of an anchor plate and shaker. The anchor plate is fixed on the shaker with a rubber sheet. The chambers are fixed on the anchor plate. (D–G) Histochemical staining of coronal sections through the parietal cortex (D) and hippocampus (E–G). (D) DAB histochemical staining indicates a focal hemorrhage (arrow) in the parietal corte × 2 h after shaking P3 rats. (E, F) Double labeling to identify extra-vessel bleeding. Endothelial cells of blood vessels are immunohistochemically stained with RECA-1 (blue), and red blood cells were stained with DAB (brown). (E) Focal hemorrhage (arrow) in the hippocampus 2 h after shaking P3 rats. (F) High magnification view of the focal hemorrhage in E. (G) High magnification view of the hippocampus stained with the modified Perls method without H_2O_2 pretreatment 2 h after shaking P7 rats. A cluster of red blood cells (arrow) and iron-positive cells (arrowheads) are evident. Scale bars = 400 μm (D, E), 100 μm (F), and 40 μm (G)..

2. Materials and methods

2.1. Animals

Timed-pregnant female Sprague-Dawley rats were purchased from Japan Charles River Lab Inc. (Tsukuba, Japan) and were housed under controlled conditions of temperature (22 ± 2 °C), humidity (50–60%), and a regular 12-h light-dark cycle with ad libitum access to food and water. The date of birth was considered postnatal day 0 (P0). All experimental procedures were performed following approval by the Animal Welfare Committee of Dokkyo Medical University School of Medicine and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Apparatus

The shaking apparatus was composed of three parts: the holding chamber for holding an individual rat pup; the anchor plate that connected the chamber and shaker; and the shaker (Fig. 1A–C). The holding chamber was a transparent cylinder made of polyvinyl chloride with an outside diameter of 40 mm, inside diameter of 38 mm, and length of 80 mm (Fig. 1A). The body of the chamber had 12 openings for ventilation. One side of the chamber was closed, and the other side opened with a hatch. At the time of shaking, the pup was fixed in the chamber with cushioning materials made of plant starch and polyvinyl alcohol (Fig. 1B). The anchor plate was a transparent plate (300 mm × 350 mm × 5 mm) made of polyvinyl chloride with attachments for 10 holding chambers at once. The shaker was a common laboratory shaker (SHK-U3, IWAKI Co. LTD., Tokyo, Japan). The pistons moved back and forth horizontally at speeds of 0–250 revolutions per minute (rpm) (Fig. 1C).

2.3. Experimental design

To evaluate the intensity of shaking, male P3-14 rat pups were anesthetized with 2% isoflurane or sevoflurane in air and were shaken for 60 s and allowed to rest for 60 s; these steps were repeated five times per day (S group). The control (C group) pups were placed in the chamber without shaking for the same experimental period under anesthesia. Based on the shaking frequencies of previous rodent models of SBS [4,5], we used 250 rpm (26.16 rad/s, 4.1 Hz) for the intensity (frequency) of shaking in this study. Throughout the experiments, to prevent hypoxia-asphyxia stress, changes in skin color and breathing were carefully observed during and after shaking [5]. After shaking, all pups were subsequently returned to their dam until weaning (P21). Offspring were then placed in a cage with 2–3 animals per cage. The time-course of the present experiment is shown in Suppl. Fig.1.

2.4. Tissue preparation and immunohistochemistry

To examine the presence of hemorrhages in the brain, 2 h after shaking P3 and P7 rats in the S group and pups in the age-matched C group were used for the first step of histological investigation. Under deep anesthesia with pentobarbital, these rats were transcardially