EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Visual and Motor Deficits in Grown-up Mice with Congenital Zika Virus Infection

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ARTICLE INFO

Article history:
Received 7 March 2017
Received in revised form 11 April 2017
Accepted 20 April 2017
Available online xxxx

Keywords:
ZIKV
Congenital Zika syndrome
Intracranial calcifications
Visual and motor dysfunctions
Prognosis evaluation

ABSTRACT

Human infants with congenital Zika virus (ZIKV) infection exhibit a range of symptoms including microcephaly, intracranial calcifications, macular atrophy and arthrogryposis. More importantly, prognosis data have lagged far behind the recent outbreak of ZIKV in 2015. In this work, we allow congenitally ZIKV-infected mice to grow into puberty. These mice exhibited motor incoordination and visual dysfunctions, which can be accounted by anatomical defects in the retina and cerebellar cortex. In contrary, anxiety level of the ZIKV-infected mice is normal. The spectrum of anatomical and behavioral deficits is consistent across different mice. Our data provided evidence that may help predict the public health burden in terms of prognosis of ZIKV-related congenital brain malformations in an animal model. Our study provided behavioral evaluation for the prognosis of congenital ZIKV infection and provides a platform for screening and evaluation of drugs candidates and treatment aiming at improving regeneration of infected neurons to prevent sequelae caused by ZIKV infection of fetus.

1. Introduction

The first human case of Zika virus (ZIKV) infection was published in 1952 (Dick, 1952). However, up to 2007, only 14 human cases of ZIKV infection had been confirmed (Fagbami, 1979; Moore et al., 1975; Olson et al., 1981). Since late 2015, ZIKV infection has swept across >35 countries and territories in Caribbean and South America (Fauci and Morens, 2016) after originating from an outbreak in Brazil (Campos et al., 2015). Although ZIKV has long been considered neurotropic (Dick, 1952; Dick et al., 1952), no specific neurological sequelae had ever been reported until this 2013–2014 epidemic during which reports linking ZIKV infection with Guillain-Barré syndrome emerged with noticeable frequency (Cao-Lormeau et al., 2016; Rasmussen et al., 2016). Of greater concern is an upsurge of cases and studies indicating a causal role between the ZIKV outbreak and an increased number of neonates in Brazil with birth defects, such as microcephaly and intrauterine growth restriction (IUGR) (Rasmussen et al., 2016; Heymann et al., 2016; Mlakar et al., 2016). However, up to now, we have gained very little knowledge about the prognosis of these children with congenital abnormalities due to the very recent outbreak of Zika.

Recent works simulated the vertical transmission of ZIKV in pregnancy and imitated its pathogenesis in fetuses (Cugola et al., 2016; Miner et al., 2016a; Wu et al., 2016; Li et al., 2016a). All the mice in these studies exhibited the microcephalic phenotype that was found in humans, and thus supported the causality between congenital ZIKV infection and microcephaly. However, none of the mice in these studies grew into puberty or adulthood either because of death of infected pups shortly after delivery (Li et al., 2016b) or the experimental necessity of ceasing the mice-rearing for further examinations. Therefore, the implications that could have otherwise been gained from these mouse models were, instead, limited to the mere link between microcephaly and ZIKV infection, not prognosis.

Although microcephaly was the most remarkable phenotype, it is not the only phenotype discovered in presumed ZIKV-infected human fetuses, as pointed out by many studies (França et al., 2016). In addition, evidence was advanced that the disease could be complicated by other clinical manifestations, such as IUGR (Brasil et al., 2016), intracranial calcification (ICC) (Oliveira Melo et al., 2016), cerebellar hypoplasia (de Fatima Vasco Aragao et al., 2016), arthrogryposis (van der Linden et al., 2016), and ocular malformations (Miranda et al., 2016), which, in the aggregate, constituted the so-called 'congenital Zika syndrome'. Among these, the most distinctive is ICC, essentially because it was extensively believed to underlie the pathogenesis of congenital ZIKV infection (Livingston et al., 2014). Vision-threatening lesions including macular lesions and optic nerve abnormalities were recently reported to be clinically associated with congenital ZIKV-infection (de Paula

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http://dx.doi.org/10.1016/j.ebiom.2017.04.029

Freitas et al., 2016). Arthrogryposis was present in the arms and legs of ZIKV-infected children (van der Linden et al., 2016). To date, these additional features show more and more conspicuous importance in the context of the ZIKV epidemic.

2. Materials and Methods

2.1. Zika Virus Preparation

Zika virus strain SZ01 (GenBank accession number: GEO: KU866423) used in this study was isolated from a Chinese male patient returning from Samoa to Shenzhen in February 2016 (Deng et al., 2016) and kindly provided by Dr. Cheng-Feng Qin. Virus stocks were amplified in mosquito C6/36 cells. The virus containing supernatants were clarified by centrifugation and stored at −80 °C. The titer of ZIKV was determined by standard plaque-forming assay on BHK21 cells. Heat inactivation of ZIKV strain SZ01 was performed at 121 °C for 30 min.

2.2. Mice and Intra-amniotic Injection (IAI)

The pregnant C57BL/6 mice (E15) were purchased from Shanghai Yison Biotechnology Company.

Pregnant mice were anesthetized during the surgery at room temperature. Mouse belly was shaved and disinfected with alcohol and iodophor. A sterile gauze was draped over the abdomen and a slit was made to expose the abdominal incision. A 3 cm long laparotomy was carefully made around the position of womb. Firstly, abdominal skin was cut open along ventral midline, and peritoneum incision was performed carefully to avoid bleeding. The uterus was pulled out carefully using tweezers and kept wet with warm, 0.1 M sterile phosphate-buffered saline (PBS). 0.1 mL 250 and 500 PFU (Plaque-Forming Units) ZIKV SZ01 was inoculated into amniotic fluid of each embryo of 3 pregnant mice (in each group) through the uterine wall with pipette (World Precision Instrument, Inc., USA) pulled by micropipette puller (Sutter Instrument, USA), respectively. The pipette stayed in amniotic fluid for 10 s after injection to avoid virus overflow. 500 PFU of heat-inactivated ZIKV were injected in each embryo of another 10 pregnant mice, referred to as mock group. The uterus was repositioned into the womb after injection and the abdominal wall and skin were stitched. Lidocaine was applied on the wound. Mice were removed into cages to recover. All mice used in the experiments were from multiple litters. The survival rate for ZIKV-infected mice was 76% (29/38 pups), whereas that for mock-infected mice was 65% (24/37 pups). The difference in the survival rate between ZIKV-infected and mock group showed no significant difference (We assumed the distribution of survival rate is independent normally distributed. In Chi-square test, $n = 75$, expected values >5, $P = 0.2762$). All mouse experimental procedures were approved by Institutional Animal Care and Use Committee at Shanghai Public Health Clinical Center. All measurements were conducted blind to the group.

2.3. RNA Extraction and Real-time Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from the brains or eyes of the pups with TRIzol Reagent (Invitrogen) according to the protocol provided by manufacturer. The RNA in mice urine was extracted by using QIAamp Viral RNA Mini Kit (Qiagen). The viral RNA copies were determined by RT-PCR (TaqMan) assay using One Step PrimeScript™ RT-PCR Kit (Takara). Primers and fluorogenic probes for ZIKV detection, ZIKV 835, ZIKV 911c and ZIKV 860-FAM were kindly provided by Prof. Yunwen Hu and Fahui Da. Reverse transcription reaction of 10 min at 42 °C was followed by PCR amplification using a 55 °C annealing temperature for 40 cycles in an ABI ViiA7 Real-Time PCR System. The standard curve of viral RNA copies was determined from 10-fold dilutions of plasmid containing ZIKV sequence (also kindly provided by Prof. Yunwen Hu and Fahui Dai) with known concentrations, and viral RNA copies were calculated.

2.4. Micro-computed Tomography (Micro-CT)

Mice were anesthetized and immobilized by adhesive tape. All micro-CT data were acquired using high-resolution, small animal imaging scanner (eXplore Locus, GE Healthcare, Port Washington, NY) with 80 kV, 450 μA and 100 ms exposure time. The detector bin mode was 4 × 4 and the effective pixel size was 0.092 mm over 360° of a total of 400 views, 3 frames per view. The raw data were converted into a VFF format compatible with MicroView ABA2.2 (GE Healthcare) to view intracranial calcification, measure the parameters of brain sizes and reconstruct 3D brains. We calculated the number of calcified foci for the whole brain and measured the lengths along both the major and perpendicular axis of the largest calcified foci of each brain in the sagittal sections. We measured the largest distances of dorsal-ventral (cranial height) and posterior–anterior (skull length) boundaries in sagittal sections, as well as medial-lateral (biparietal) in coronal sections. To calculate the brain volumes, we manually selected one out of every 5 sagittal sections in 2D Region of Interest (ROI) and reconstructed all the 2D images into 3D using the Advanced ROI Tool in MicroView ABA2.2.

2.5. Open Field Test

The open field (OF) test was used to assess the adaption and anxiety-like behavior as described previously (Blokland et al., 2002). Each mouse was placed in the center of a darkened white box (30 cm × 30 cm × 40 cm) and monitored by an infrared video tracking system for 25 min (Ethovision XT 9.0, Noldus Information Technology, The Netherlands). A 15 cm × 15 cm square in the center of the box was defined as Zone and the periphery arena as Residual. The distance traveled and time spent in the Zone and Residual were quantified for analysis.

2.6. Tail Suspension Test

Each mouse was suspended from a hanging hook located 45 cm above the bottom of the box using adhesive tape placed approximately 1 cm from the tip of the tail. Each mouse was tested for 6 min and recorded by a digital camera. Software (Ethovision XT 9.0, Noldus Information Technology, The Netherlands) was used to calculate the inactive (hanging passively without any struggle) time of each mouse.

2.7. Rota-rod Test

Each mouse was placed on a rotating spindle that accelerated from 4 revolutions per minute (rpm) to 40 rpm in 5 min (Rotamex-5, Columbus Instruments, Columbus, OH). All animals did one session per day for 3 days, and the latency to falling off the spindle in each session was recorded for analysis.

2.8. CatWalk

Each mouse walked freely on a glass lit by green light in the CatWalk system (CatWalk XT 10.0, Noldus Information Technology, The Netherlands). The gait and paw print images of each mouse were recorded by a high-speed camera for at least 3 sessions. Walking patterns and postures were analyzed.

2.9. Elevated Plus Maze

The apparatus consisted of two open arms (30 cm × 5 cm, 350 lx) and two close arms (30 cm × 5 cm × 15 cm, 20 lx), 50 cm from the ground. Each mouse was placed in the central platform (5 cm × 5 cm)

Please cite this article as: Cui, L., et al., Visual and Motor Deficits in Grown-up Mice with Congenital Zika Virus Infection, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.04.029