ZIKA VIRUS IMPAIRS NEUROBEHAVIORAL DEVELOPMENT AND INDUCES OXIDATIVE STRESS LINKED TO BLOOD–BRAIN BARRIER DISRUPTION IN A RAT MODEL OF CONGENITAL INFECTION

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https://doi.org/10.5281/zenodo.14783854

Introduction

Zika virus (ZIKV) is an arbovirus belonging to the *Flaviviridae* family and the *Flavivirus* genus, which caused a significant epidemic in the Americas in 2015 (Song et al., 2017). Gestational ZIKV infection was soon linked to neurodevelopmental complications in fetuses, leading to various adverse outcomes such as intrauterine growth restriction, fetal death, miscarriage, stillbirth, and ocular abnormalities (Alvarado & Schwartz, 2017; Coyne & Lazear, 2016). Among these, microcephaly emerged as the most prominent clinical manifestation and is now well-established as a consequence of ZIKV vertical transmission (Wang & Ling, 2016; Li et al., 2016). While microcephaly was initially considered the primary neurological outcome of congenital ZIKV infection, recent studies have demonstrated that children affected by ZIKV in utero, even without microcephaly, may still exhibit neurodevelopmental delays (Sobral da Silva et al., 2021). Furthermore, growing evidence suggests that ZIKV-induced pathogenesis extends beyond the immediate postnatal period, with potential long-term effects on neurological development.

Several models have been developed to explore the pathophysiology of ZIKV infection and its impact on the nervous system. *In vitro* studies have been instrumental in elucidating the cellular and molecular mechanisms underlying ZIKV-induced neurological damage. For instance, studies using U87-MG (human glioblastoma) and HepG2 (human liver carcinoma) cell lines have revealed that ZIKV infection leads to increased reactive oxygen species (ROS), lipid peroxidation, and protein carbonylation, alongside a decrease in antioxidant enzyme activity, including superoxide dismutase and catalase (Almeida et al., 2020). Similarly, human neural progenitor cells (hNPCs) infected with ZIKV exhibited cell cycle dysregulation and increased caspase-3 activation, ultimately leading to cell death (Tang et al., 2016). In radial glial cells (RGCs), ZIKV infection disrupts mitosis and structural organization and leads to the sequestration of phosphorylated TBK1 during mitosis (Onorati et al., 2016). Given the crucial role of RGCs in cortical cell migration, these findings help explain the cortical thinning observed in ZIKV-infected models.

Another significant consequence of ZIKV infection involves damage to the blood–brain barrier (BBB), a critical structure that regulates the exchange between systemic circulation and the central nervous system (Clé et al., 2020). Studies conducted *in vitro* and *in vivo* have demonstrated that ZIKV infection disrupts BBB integrity (Leda et al., 2019). However, it remains unclear whether these alterations are transient or contribute to long-term neuropathological outcomes. Additionally, the full spectrum of BBB-related damage induced by ZIKV infection has yet to be fully elucidated.

Regarding *in vivo* studies, various rodent models have been employed to investigate ZIKV pathogenicity, fetal infection, and vertical transmission. Since wild-type (WT) rodents

exhibit resistance to ZIKV due to their robust interferon (IFN) response, many studies have relied on IFN-deficient animal models to assess infection-related outcomes (Kublin & Whitney, 2018). Immunocompromised mice, such as A129 and AG129 strains, have been instrumental in understanding the impact of vertical transmission (Vue & Tang, 2021). Additionally, alternative models have explored different routes of infection, including direct intrauterine exposure to the developing rodent brain (Li et al., 2016) and postnatal infection shortly after birth (Lazear et al., 2016; Miner et al., 2016). Given the need to better understand immune responses, long-term consequences, and potential therapeutic interventions, further research involving immunocompetent animal models is essential (Morrison & Diamond, 2017).

Methods

Animal Procedures

This study utilized pregnant female Wistar rats, approximately two months old, obtained from the Animal Reproduction and Experimental Center at the Federal University of Rio Grande do Sul, Brazil. The animals were housed in individually ventilated cages (IVC) under a 12-hour light/dark cycle, with a controlled temperature of 21 ± 2°C, and provided with unrestricted access to food and water. All experimental procedures adhered to the ethical guidelines established by the National Council for Animal Experimentation of Brazil and complied with Brazilian legislation for the scientific use of animals (Law 11.794/08). Additionally, procedures followed the *Guide for the Care and Use of Laboratory Animals* as outlined by the National Research Council (USA, 2011).

Ethical approval for this study was granted by the Ethics Committee of the Federal University of Rio Grande do Sul (approval number 33452/2017). All procedures were conducted in a Biosafety Level 2 (BSL-2) laboratory under Animal Biosafety Level 2 (ABSL-2) conditions, in accordance with the guidelines of the Centers for Disease Control and Prevention (CDC) for laboratory work involving Zika virus. Virus handling and animal-related procedures were performed within a Class II Biological Safety Cabinet (BSC) (Tecniplast® BS 60 class II, Buguggiate, Italy) to ensure biosafety compliance.

On embryonic day 9 (E9), pregnant females were randomly assigned to two groups. Five females received an intraperitoneal injection of 500 µL containing 1×10^6 plaque-forming units per milliliter (PFU/mL) of Zika virus isolated in Brazil (*ZIKV_BR*). The control group consisted of six females that received a 500 µL intraperitoneal injection of sterile diluted medium.

Neurological Reflexes

Twenty-four hours after birth, litter standardization (5–8 pups per litter) and body measurements were conducted (Table 1). To assess neurodevelopmental impairments associated with gestational Zika virus infection, a series of neurological reflex evaluations were performed. These assessments began on postnatal day (PND) 3 and were conducted every three days until PND 21. Observations included physical characteristics such as body weight, eye opening, and incisor tooth eruption (Lubics et al., 2005).

The following reflexes and motor responses were evaluated:

- **Righting Reflex:** Each pup was placed on its back, and the time taken to return to a prone position with all four paws in contact with the surface was recorded.

- **Negative Geotaxis:** Pups were positioned on an inclined platform (45°) with their heads facing downward. The day when they were able to turn their heads upward and climb the platform was noted, with a maximum time limit of 30 seconds to complete the task.
- **Limb Placing:** The posterior region of the forepaw and hind paw was gently pressed against a surface, and the day when the pup successfully placed its paws onto the surface was recorded.
- **Limb Grasp:** The back of the forelimb paws was lightly touched against a stem, and the day when the pup grasped the stem was noted.
- **Cliff Aversion:** Each pup was placed with its head near the edge of an elevated platform, and the time taken to turn its head away from the edge was measured. Additionally, the first day this behavior was observed was recorded.
- **Gait Assessment:** Pups were placed at the center of a 30 cm-diameter circle, and the time taken to exit the circle was recorded, along with the first day they exhibited locomotor movement.

Results

Day of Appearance of Neurological Reflexes

To assess the emergence of neurological reflexes, a **t-test** was conducted, comparing male ZKV (Zika virus-infected) with male CT (control) and female ZKV with female CT. A significant delay in the onset of specific neurological reflexes was observed in male ZKV compared to male CT in the following parameters:

- **Incisor tooth eruption** *($t(28) = 5.82; P < 0.05$)*
- **Forelimb placing (right)** *($t(28) = 4.58; P < 0.05$)*
- **Hind limb placing (right)** *($t(28) = 5.15; P < 0.05$)*
- **Hind limb placing (left)** *($t(28) = 3.35; P < 0.05$)*
- **Forelimb grasp (right)** *($t(28) = 2.15; P < 0.05$)*

No significant differences were detected in the timing of **eye opening, negative geotaxis, forelimb placing (left), forelimb grasp (left), gait, aversion to fall, righting reflex, or olfactory behavior**.

For females, a similar analysis revealed delayed reflex development in the following:

- **Hind limb placing (right)** *($t(13) = 3.10; P < 0.05$)*
- **Hind limb placing (left)** *($t(13) = 4.56; P < 0.05$)*
- **Righting reflex** *($t(28) = 2.28; P < 0.05$)*

No significant differences were found in other assessed reflexes.

Overall, these findings indicate that gestational Zika virus infection resulted in delays in the appearance of certain neurological reflexes in both male and female offspring. These delays may serve as early indicators of potential long-term neurological alterations.

Data are presented as **mean ± SD**. *(ZKV)* refers to offspring from Zika virus-infected mothers, while *(CT)* represents control animals. Sample sizes: Female CT *($n = 8$)*, Female ZKV *($n = 7$)*, Male CT *($n = 15$)*, Male ZKV *($n = 15$)*. Asterisks (*) indicate significant differences compared to the control group of the same sex (Student's t-test, $p < 0.05$).

Discussion

In this study, we expanded the understanding of congenital Zika virus (ZIKV) syndrome by developing a rat model of congenital ZIKV infection to investigate neurodevelopmental

impairments and brain tissue disturbances in Wistar rats shortly after birth. The infection was induced on embryonic day 9 (E9), a crucial stage for rodent neurodevelopment (Semple et al., 2013). Pregnant rats received an intraperitoneal (i.p.) injection of ZIKV, and viral presence was detected in maternal blood as early as six hours post-infection. Additionally, viable virus was found in the placenta, spleen, and fetuses within 24 hours post-inoculation. Notably, infected females did not exhibit sickness behavior despite their offspring showing neurological impairments, which aligns with previous studies (Sherer et al., 2019). This finding is particularly relevant, given that asymptomatic ZIKV circulation has been reported among pregnant women in northeastern Brazil (Branco et al., 2021). Furthermore, even asymptomatic maternal ZIKV infection has been associated with neurodevelopmental impairments in offspring (Shapiro-Mendoza et al., 2017). Consistently, our study identified significant neurobehavioral deficiencies in infected pups, suggesting potential long-term cognitive and motor disturbances. On postnatal day 22 (PND 22), blood–brain barrier (BBB) integrity deficits in the hippocampus and altered oxidative status in the hippocampus and cortex were observed, highlighting the potential for lasting structural and functional brain damage.

Conclusion

This study sought to deepen the understanding of the long-term effects of gestational Zika virus (ZIKV) infection by developing an immunocompetent rat model and identifying neurobehavioral markers predictive of future disabilities. Our findings reveal that, even in the absence of brain morphometric changes or a microcephaly-like phenotype, infected offspring exhibited significant neurodevelopmental impairments. These deficits were associated with hippocampal and cortical blood–brain barrier (BBB) disruption and oxidative stress imbalance observed 22 days postnatally.

Together, these results provide strong evidence that the impact of gestational ZIKV infection extends beyond the neonatal period, with potential long-term consequences for brain function and development. Our study contributes to existing knowledge by highlighting the need for improved prenatal diagnostics, early interventions, and extended follow-up studies to further elucidate the mechanisms underlying congenital ZIKV syndrome. Furthermore, the development of a reliable congenital infection model not only enhances our understanding of disease pathology but also offers new opportunities for advancing therapeutic strategies.

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