The Journal of Infectious Diseases

MAJOR ARTICLE

Can We Better Understand How Zika Leads to Microcephaly? A Systematic Review of the Effects of the Zika Virus on Human Brain Organoids

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Background. The innovative human brain organoid model represents a unique opportunity to better understand the genesis of congenital brain abnormalities, particularly microcephaly, caused by Zika virus (ZIKV) infection during early pregnancy.

Methods. A systematic review was conducted to investigate how ZIKV leads to microcephaly in a novel experimental model that mimics early brain development. Studies were gathered by searching MEDLINE/Pubmed, LILACS, and LiSSa for reports on effects of ZIKV infection on human brain organoids. From 146 identified papers, 13 articles were selected for review.

Results. This review found that ZIKV of African, Latin American, and Asian lineages caused productive replication after 72 hours, preferentially infected neural progenitor cells over mature neurons, reduced both cell populations, and caused premature differentiation. Limited data involving only African and Latin American lineages showed a reduction in populations of proliferating cells and intermediate cells, and overall decreased viability. Furthermore, all 3 lineages caused heightened apoptosis and reduced organoid size.

Conclusions. This review concludes that, in organoids, ZIKV causes productive replication, infects neural progenitor cells over mature neurons, decreases both populations, causes premature differentiation, induces apoptosis, and reduces size.

Keywords. Zika; organoid; microcephaly; neural progenitor cells.

Studying the effects of the Zika virus (ZIKV), a member of the Flaviviridae family, during early human brain development is challenging. Animal models have limitations. For example, mouse cortical development differs from human as it includes different cell populations and antiviral signaling pathways [1], while nonhuman primate models like macaques are seasonal breeders, rendering the experimental process very time consuming [2].

The use of human brain organoids represents a novel method to investigate early brain development akin to early first trimester in humans, a critical period when the maternal decidua, fetal placenta, and umbilical cord are especially permissive to ZIKV infection [3, 4]. Organoids are 3-dimensional cell aggregates generated in vitro from human pluripotent stem cells [5]. They contain a ventricular zone, with NESTIN-, SOX2-, PAX6-, or phosphovimentin-positive neural progenitor cells at the apical side facing a lumen, and CTIP2- or TUJ1-positive mature neurons at the basal side [6]. TBR1- or TBR2-positive cells are intermediate progenitor cells found prominently in the subventricular zone. Cell proliferation is measured by Ki67 marker or phosphate-H3, while cell viability or cell death is determined by 5-ethynyl-2′-deoxyuridine (EdU) labeling. Apoptotic cells are measured using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or caspase-3 (CASP3) assays.

In the developing human cerebral neocortex, neuroepithelial stem cells transition into neural progenitor cells, also known as radial glia cells, and reside in the ventricular and subventricular zones, giving rise to intermediate progenitors before becoming neurons, astrocytes, and oligodendrocytes [7, 8]. Recently, it was shown that ZIKV targets neuroepithelial stem cells and neural progenitor cells, impairing mitosis and survival [9].

Human brain organoids provide a unique opportunity to investigate the causal relationship between ZIKV and microcephaly in a controlled setting. Thus, this systematic review aimed to address the following: what are the effects of ZIKV infection on human brain organoids, which cells are most vulnerable, and is there a difference in outcome among ZIKV lineages?

METHODS

Protocol and Registration

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist, found in Supplementary

Received 20 May 2018; editorial decision 18 September 2018; accepted 22 September 2018; published online September 26, 2018.

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The Journal of Infectious Diseases® 2019;219:734–45

DOI: 10.1093/infdis/jiy572

Table 1, was adhered to for this systematic review. The protocol was not registered.

Eligibility Criteria

Inclusion Criteria

Articles were included that studied the effects of ZIKV on human cerebral or region-specific organoids, regardless of cell lines used for its generation (ie, human embryonic stem cells or induced pluripotent stem cells).

Exclusion Criteria

Studies were excluded if: (1) organoids were not of human origin; (2) organoids were not of brain origin; (3) organoids differentiated to tumor progeny; (4) reviews of literature, protocol summaries, editorials, opinions pieces, conference abstracts, and book chapters; or (5) clinical studies.

Information Sources and Search Strategies

A comprehensive literature search using MEDLINE/PubMed, LiSSa (Littérature Scientifique en Santé), and LILACS (Literatura Latino Americana em Ciências da Saúde) electronic databases was conducted up to and including 1 February 2018. The terms "Zika," "ZIKV," or "flavivirus" were selected in combination with the words "organoid," "in vitro," "culture," "3D" (from 3-dimensional culturing), "bioreactor" (a technique for organoid generation), and "stem cells," "progenitors," "HESC," or "IPSC" (sources of derivations of the organoids) for the complete search strategy, which is found in Supplementary Material 2.

Study Selection

Initial triage of articles was based on whether titles or abstracts met the inclusion criteria. Full-text articles and supplementary material were then read, and those that did not satisfy the inclusion/exclusion criteria were excluded.

Data Collection Process and Data Items

Data extracted from articles or supplementary material included the name of first author, year of publication, country, and study design. Variables for which the data were sought included the age and type of experimental model (ie, cerebral or region-specific organoids), ZIKV strain, protocol of infection including duration of effects and multiplicity of infection (MOI) or viral dilution, physical changes of organoids following ZIKV infection, and effects of specific types of cells based on markers used, including cell death and cell viability. Statistical data were gathered only if measurements were compared to mock-infected controls.

Risk of Bias in Individual Studies and Across Studies

The National Toxicology Program Office of Health Assessment and Translation (OHAT) Risk of Bias Rating Tool for Human and Animal Studies by the US Department of Health and Human Services was used to methodically appraise the quality of evidence of all studies. The methodology is found in Supplementary Material 3. Potential bias across studies were analyzed within study characteristics.

Synthesis of Results and Summary Measures

Viral replication and preferential infectivity, cell population, activity, and death, and organoid characteristics following ZIKV exposure were tabulated, evaluated, and summarized.

RESULTS

Study Selection

From 3 databases146 articles were selected. Based on relevance to ZIKV and in vitro human brain organoid experimentation, 27 articles were selected. Only 13 articles fully complied with the study selection criteria [10–22]. A PRISMA flow diagram detailing the process of identification, inclusion, and exclusion of studies is shown in Figure 1.

Study Characteristics

A summary of study characteristics is given in Table 1. All studies were published between 2016 and 2017. The majority of studies were conducted in the United States [11, 15, 16, 18, 20–22], while 3 took place in Brazil [10, 13, 17]. Germany [12] and China [14] contributed 1 study each. Organoid characteristics and infection protocol are given in Supplementary Material 4.

Strains

Five different strains were used. The majority of articles [10–13, 15, 16, 21, 22] used MR766 from Uganda (African lineage). FSS13025 from Cambodia (Asian lineage) was used in 2 articles [16, 20]. Contemporary Latin America strains (Latin American lineage) were isolated from different origins. PRVABC59, used in 3 studies [14, 18, 19], originated from Puerto Rico. Although 3 studies [10, 12, 17] sourced their strains from patients in Brazil, the strain used by Gabriel et al [12] originated from fetal brain exhibiting neurological abnormalities. Only 1 study [12] used H/PF/2013 originating from French Polynesia (included as part of the Asian lineage for data assessment purposes).

Risk of Bias

All articles were rated as high quality, whereby questions concerning "exposure characterization" and "outcome assessment" were assessed as very low risk of bias and the majority of remaining questions rated as low risk of bias. However, perceived biases were present. Eleven studies did not blind their research personnel to their respective groups [10–16, 19–22], while 6 studies had incomplete data. Gabriel et al [12] omitted measurements for 1 or 2 out of 3 ZIKV strains for cortical plate thickness, organoid diameter, and TUJ1-positive cells within the ventricular zone lumen. In addition, values for TUNEL assay were not presented. Studies by Dang et al [11], Li C et al [14], and Sacramento et al [17] did not provide sample size for all experiments despite calculating statistical results. Also,

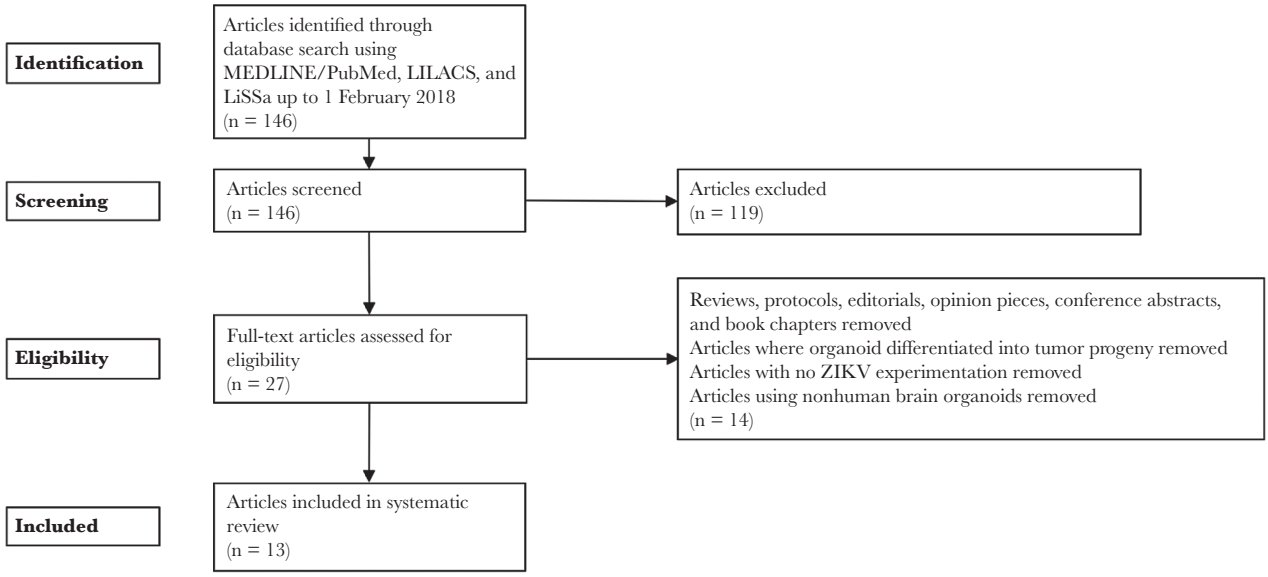

Figure 1. Flow diagram of literature search and selection criteria adapted from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol.

Sacramento et al [17] did not use mock-infected controls. Li Y et al [15] and Qian et al [16] had neither provided the number of organoids used for each experiment, nor disclosed the full statistical method. Furthermore, Li Y et al [15] did not quantify apoptosis measurements by CASP3 assay, while Qian et al [16] did not report the results for all strains in SOX2- and TBR2-positive cell measurements. Details of the Office of Health Assessment and Translation risk of bias evaluation are found in Table 2.

Results of Individual Studies

A summary of findings is given in Table 3.

Comparison of ZIKV Strains

Three articles investigated the effects of different ZIKV strains on human brain organoids. Cugola et al [10] demonstrated that both Latin American and African strains reduced proliferative zones and disrupted cortical layers. Using strains from Asia, Latin America, and Africa, Gabriel et al [12] stated that they all infected neural progenitor cells causing premature differentiation. Finally, Qian et al [16] discovered preferential and productive infection of neural progenitor cells with either African or Asian strains leading to increased cell death and reduced proliferation.

ZIKV Pathogenesis

Five articles studied ZIKV pathogenesis. Garcez et al [13] was one of the first to observe ZIKV-mediated reduction of viability and growth of human brain cell. Dang et al [11] recorded ZIKV activation of *TLR3*, an innate immune receptor, which depleted neural progenitor cells. Yoon et al [21] introduced the ZIKV-encoded nonstructural protein NS2 into organoids, resulting in reduced neural progenitor cell proliferation and adherens junction deficits. Wells et al [19] discovered that genetically ablating *AXL*, considered the ZIKV entry receptor found on neural progenitor cells, had no effect on ZIKV entry or ZIKV-mediated cell death. Finally, Li Y et al [15] reported that ZIKV impaired cortical growth and folding by interacting with the *PTEN* gene.

Drug Candidates That Inhibit ZIKV Infection

Five articles examined drugs with the potential to inhibit ZIKV infection. Xu et al [20], Watanabe et al [18], and Zhou et al [22] used organoids to identify small-molecule candidates to combat ZIKV infection. Sacramento et al [17] analyzed sofosbuvir, while Li C et al [14] tested 25-hydroxycholesterol, an amplifier of inflammatory signaling.

Synthesis of Results

The effects on human brain organoids by ZIKV strains, grouped according to lineage (ie, African, Latin American, and Asian), are summarized in Table 4.

Viral Copies and Preferential Infectivity

Six articles quantified ZIKV numbers from organoid supernatant using quantitative real-time polymerase chain reaction [11, 12, 14, 16, 17, 22]. All lineages showed increasing viral numbers after 72 hours. ZIKV replication was apparent 12 hours postexposure ($P < .001$ unpaired t test vs 0 hours) [14].

ZIKV preferentially infected neural progenitor cells over neuronal cells, followed last by intermediate cells [12, 16, 18]. A comparison of strains by Qian et al [16] showed that the Asian strain was found predominantly in SOX2-positive progenitor cells (90%) followed by CTIP2-positive neuronal cells (5%) and TBR2-positive intermediate cells (5%), while the majority of

Table 1. Summary of Descriptive Characteristics of Included Articles (n = 13)

Reference, Publication Year	Country, Study Design	Experimental Model, Age of Organoid	ZIKV Strain(s); MOI or Dilution; Duration of Infection	Main Conclusion
Cugola et al [10], 2016	Brazil, in vitro experimentation	Human cerebral organoids, 28 d	MR766 (ZIKV-AF), Brazilian (ZIKV-BR); MOI 0.1; 24 h	The infection of cerebral organoids by ZIKV-BR or ZIKV-AF results in a reduction of proliferative zones and disrupted cortical layers, which reinforces evidence linking ZIKV-BR outbreak to congenital brain malformations.
Dang et al [11], 2016	United States, in vitro experimentation	Human cerebral organoids, 10 d	MR766 (ZIKV-AF); MOI 1; 24 h	ZIKV strain MR766 efficiently infects cerebral organoids and causes a decrease in overall organoid size that correlates with the kinetics of viral copy number. A link is identified between ZIKV-mediated *TLR3* activation, perturbed cell fate, and a reduction in organoid volume reminiscent of microcephaly.
Gabriel et al [12], 2017	Germany, in vitro experimentation	Human brain organoids, 9 d	MR766 (ZIKV-AF), FB-GWUH-2016 (ZIKV-AM),H/PF/2013 (ZIKV-AS); MOI 1; 2 d, 5 d, or 11 d	Two recently isolated strains, ZIKV-AM and ZIKV-AS, efficiently infect neural progenitors in brain organoids, causing premature differentiation resembling ZIKV-associated microcephaly. The effects are different from that seen with ZIKV-AF, an older and extensively passaged strain.
Garcez et al [13], 2016	Brazil, in vitro experimentation	Human brain organoids, 35 d	MR766; MOI 0.0025–0.25 (1:100, 1:1000 dilution); 2 h	ZIKV targets human brain cells, reducing their viability and growth as brain organoids, suggesting that ZIKV abrogates neurogenesis during human brain development.
Li C et al [14], 2017	China, in vitro experimentation	Human cortical-specific organoids, 23 d	PRVABC59; MOI 0.3–3.12 (1:2–1:8 dilution); 2 h	25HC (25-hydroxycholesterol) inhibits ZIKV infection in human cortical organoids and has the potential as a first-line antiviral agent to combat ZIKV.
Li Y et al [15], 2017	United States, in vitro experimentation	Human cerebral organoids, 19 d or 30 d	MR766; MOI 1; 24 h	Infection of cerebral organoids with ZIKV impairs cortical growth and folding.
Qian et al [16], 2016	United States, in vitro experimentation	Human forebrain-specific organoids, 14 d or 28 d	MR766 (African), FSS13025 (Asian); 1:10 dilution for 14 d organoids, 1:10 or 1:40 dilution for 28 d organoids; 24 h	There is preferential and productive infection of neural progenitors in forebrain-specific organoids with either African or Asian ZIKV strains leading to increased cell death and reduced proliferation, resulting in decreased neuronal cell-layer volume resembling microcephaly.
Sacramento et al [17], 2017	Brazil, in vitro experimentation	Human brain organoids, age not available	Brazilian; MOI 1.0 or 10; 2 h	Sofosbuvir inhibited ZIKV replication in brain organoids and induced an increase in A-to-G mutations in the viral genome.
Watanabe et al [18], 2017	United States, in vitro experimentation	Human cortical-specific organoids, 21 d or 56 d	PRVABC59; MOI 0.3–3.125 (1:2–1:8 dilution); 2 h	An optimized organoid culture method that efficiently and reliably produce cortical and basal ganglia structures similar to those in the human fetal brain in vivo models the teratogenic effects of ZIKV on the developing brain.
Wells et al [19], 2016	United States, in vitro experimentation	Human cerebral organoids, 24 d	PRVABC59; MOI 0.1, 1, or 10; 2 h	Genetic ablation of *AXL* has no effect on ZIKV entry or ZIKV-mediated cell death in cerebral organoids.
Xu et al [20], 2016	United States, in vitro experimentation	Human forebrain-specific organoids, 18 d	FSS13025; MOI 0.04 to 0.08; 24 h	A pan-caspase inhibitor, emricasan, inhibited ZIKV-induced increases in caspase-3 activity and protected forebrain-specific organoids.
Yoon et al [21], 2017	United States, in vitro experimentation	Human forebrain-specific organoids, 45 d	MR766; not available; coexpression of ZIKV NS2A in radial glia cells	Systematically introducing individual proteins encoding ZIKV-NS2 in forebrain-specific organoids reduces radial glial cell proliferation and causes adherens junction deficits.
Zhou et al [22], 2017	United States, in vitro experimentation	Human forebrain-specific organoids, 20 d	MR766; MOI 0.125; 24 h	Hippeastrine hydrobromide rescued a ZIKV-induced growth and differential defects in forebrain-specific organoids.

Abbreviations: MOI, multiplicity of infection; ZIKV, Zika virus.

SOX2-positive cells (92.5%) were afflicted by the African strain followed by CTIP2- (5%) and TBR2-positive cells (2.5%).

Cell Population

Five articles showed that ZIKV reduced the neural progenitor cell population [10–12, 18, 21] in comparison to mock-infected controls, while 3 articles revealed decreased mature neuronal population [10, 12, 18]. Two articles studying the African and Latin American strains reported a reduction in intermediate cell population [10, 18]. Closer investigation revealed varying strain-dependent effects.

Cugola et al [10] reported that the Latin American strain caused greater reductions in SOX2-positive progenitor (−100%), TBR1-positive intermediate (−75%), and CTIP2-positive neuronal (−75%) cell populations than the African strain equivalent (−50%, −50%, and −25%, respectively) when compared to mock-infected controls after 96 hours. In contrast, Gabriel et al [12] revealed greater reduction in phosphovimentin-positive

Table 2. Judgment of the Quality of Evidence for Intervention Using Office of Health Assessment and Translation Risk of Bias Tool

Reference	Randomization: Was ZIKV Exposure Adequately Randomized?	Allocation Concealment: Was the Allocation of ZIKV Exposure Adequately Concealed to Researchers?	Experimental Conditions: Were the experimental Conditions Identical Across Study Groups?	Blinding During Study: Were the Research Personnel Blinded to the Study Group During the Study?	Incomplete Data: Were the Outcome Data Complete Without Attrition or Exclusion From Analysis?	Exposure Characterization: Can we be Confident in the Exposure Assessment?	Outcome Assessment: Can we Be Confident in Outcome Assessment?	Reporting: Were All Measured Outcomes Reported?
Cugola et al [10]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Low risk of bias: insufficient information provided about loss of organoids	Very low risk of bias	Very low risk of bias	Very low risk of bias
Dang et al [11]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	High risk of bias: insufficient information provided about number of organoids (number of organoids not recorded except for volume experiment)	Very low risk of bias	Very low risk of bias	Very low risk of bias
Gabriel et al [12]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	High risk of bias: cortical plate thickness measured only for ZIKV-AS, not ZIKV-AM or ZIKV-AF; values not shown for TUNEL assay; organoid diameter measured for ZIKV-AF only, not ZIKV-AS or ZIKV-AM; percentage of ventricular zone lumen exhibiting TUJ1-positive cells measured for ZIKV-AS and ZIKV-AM, not ZIKV-AF	Very low risk of bias	Very low risk of bias	Very low risk of bias
Garcez et al [13]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Low risk of bias: insufficient information provided about number of organoids	Very low risk of bias	Very low risk of bias	Very low risk of bias
Li C et al [14]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	High risk of bias: insufficient information provided about number of organoids (number of organoids not recorded)	Very low risk of bias	Very low risk of bias	Very low risk of bias

Table 2. Continued

Reference	Randomization: Was ZIKV Exposure Adequately Randomized?	Allocation Concealment: Was the Allocation of ZIKV Exposure Adequately Concealed to Researchers?	Experimental Conditions: Were the experimental Conditions Identical Across Study Groups?	Blinding During Study: Were the Research Personnel Blinded to the Study Group During the Study?	Incomplete Data: Were the Outcome Data Complete Without Attrition or Exclusion From Analysis?	Exposure Characterization: Can we be Confident in the Exposure Assessment?	Outcome Assessment: Can we Be Confident in Outcome Assessment?	Reporting: Were All Measured Outcomes Reported?
Li Y et al [15]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	High risk of bias: insufficient information provided about number of organoids (number of organoids not recorded); apoptosis measurements by CASP3 not quantified; full statistical method not disclosed for each experiment	Very low risk of bias	Very low risk of bias	Very low risk of bias
Qian et al [16]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very low risk of bias	High risk of bias: insufficient information provided about number of organoids; ZIKV-C strain limited to SOX2 and TBR2 staining; statistical method not reported	Very low risk of bias	Very low risk of bias	Very low risk of bias
Sacramento et al [17]	High risk of bias: no controls	High risk of bias: no controls	Very low risk of bias	Very low risk of bias	High risk of bias: insufficient information provided about number of organoids (number of organoids not recorded)	Very low risk of bias	Very low risk of bias	Very low risk of bias
Watanabe et al [18]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very low risk of bias
Wells et al [19]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Low risk of bias: insufficient information provided about number of organoids; Ki67 measurements not quantified	Very low risk of bias	Very low risk of bias	Very low risk of bias
Xu et al [20]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Low risk of bias: insufficient information provided about number of organoids	Very low risk of bias	Very low risk of bias	Very low risk of bias

Table 2. Continued

Reference	Randomization: Was ZIKV Exposure Adequately Randomized?	Allocation Concealment: Was the Allocation of ZIKV Exposure Adequately Concealed to Researchers?	Experimental Conditions: Were the experimental Conditions Identical Across Study Groups?	Blinding During Study: Were the Research Personnel Blinded to the Study Group During the Study?	Incomplete Data: Were the Outcome Data Complete Without Attrition or Exclusion From Analysis?	Exposure Characterization: Can we be Confident in the Exposure Assessment?	Outcome Assessment: Can we Be Confident in Outcome Assessment?	Reporting: Were All Measured Outcomes Reported?
Yoon et al [21]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very low risk of bias
Zhou et al [22]	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very high risk of bias: no evidence of blinding to study group involving organoids, author indicated that investigators were not blinded to allocation during experiments and outcome assessment	Very low risk of bias	Very low risk of bias	Very low risk of bias	Very low risk of bias

Abbreviations: AF, African; AM, American; AS, Asian; CASP3, caspase-3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ZIKV, Zika virus.

progenitor cell population after 11 days of infection for the African strain (−93%, $P < .001$ 2-way ANOVA vs mock-infected controls) than either the Latin American or Asian strain (−83% for both, $P < .001$ 2-way ANOVA vs mock-infected controls). Furthermore, the African strain infected cells distant from SOX2- or PAX6-positive progenitor cells and in greater numbers outside of the ventricular zone, whereas the Latin American and Asian strains remained localized in SOX2- and PAX6-positive cells within the ventricular zone and reduced TUJ1-positive neuronal cells at the organoid surface.

As a sign of cell cycle disruption in studies using the African strain, Zhou et al [22] observed that SOX2- and TUJ1-positive cells were intermixed and lacked discernible organization, while Yoon et al [21] found a 27.5% increase in PAX6-positive progenitor cells with abnormal multipolar morphology ($P < .01$ *t* test).

Cellular Activity

Four articles [10, 16, 21, 22] investigating cellular activity measured decreased numbers of proliferating cells in comparison to mock-infected controls. Measuring Ki67-positive cells, Zhou et al [22] detected a 10% decrease ($P < .05$ unpaired 2-tailed *t* test), while Yoon et al [21] found a 20% decrease ($P < .01$ *t* test). Measurements of phosphate-H3–positive cell density showed even greater reduction (−87.5%, $P < .0005$ *t* test) [16].

The African strain decreased Ki67- [10, 21, 22] and phosphate-H3–positive [16] proliferating cell populations in comparison to mock-infected controls, while the Latin American strain resulted in similar reductions in Ki67-positive cells [10]. No studies were conducted using strains from Asia. Comparing lineages showed greater reduction from the Latin American strain (−90%) over the African strain (−80%) after 96 hours [10].

Gabriel et al [12] was the only article to investigate ZIKV-mediated change in mitotic activity. They found elevated numbers of dividing cells exhibiting vertical orientation of the division plane, an indication of premature differentiation, after 5 days of ZIKV infection (+150% for all lineages, $P < .001$ 2-way ANOVA) when compared to mock-infected controls.

Cell Death

Two studies reported a drop in viable cells by the African strain using EdU activity in comparison to mock-infected controls. Yoon et al [21] found fewer EdU-positive total viable cells and PAX6-EdU–positive viable progenitor cells (−17.5%, $P < .001$ *t* test), while Qian et al [16] showed less EdU-positive viable cells within the ventricular zone (−28% at 0.25 dilution, $P < .005$ *t* test).

Seven articles measured apoptosis. ZIKV increased CASP3 [10, 16, 18–20] or TUNEL activity [10, 12] when compared to mock-infected controls. Xu et al [20] measured the lowest severity (+1.75%, $P < .001$ 1-way ANOVA), whereas Zhou et al [22] and

Table 3. Summary of Findings by Any ZIKV Strain for Included Articles (n = 13)

Reference	Viral Replication (Any Period)	Progenitor Cells/Radial Glia Cells (SOX2+, PAX6+, Nestin+, Phospho-vimentin+)	Intermediate Cells (TBR1+/TBR2+)	Proliferating Cells (Ki67+, Phospho-H3)	Mature Neurons (CTIP2+, TUJ+)	Apoptosis (TUNEL+, CASP3+)	Viability (EdU+)	Change in Size, Area, Volume, or Thickness	Other
Cugola et al [10]	NA	↓	↓	↓	↓ (After 96 h only)	↑	NA	↓	NA
Dang et al [11]	↑	↓	NA	NA	NA	NA	NA	↓	Upregulation of *TLR3* mRNA levels. Downregulation of *Ntn1* and *Ephb2* genes.
Gabriel et al [12]	↑	↓	NA	NA	↓	↑	NA	↓	Decreased percentage of cells showing horizontal orientation Increased percentage of cells showing vertical orientation
Garcez et al [13]	NA	NA	NA	NA	NA	NA	NA	↓	NA
Li C et al [14]	↑	NA	NA	NA	NA	NA	NA	NA	Increased expression of *IFN-B* and *CH25H* genes
Li Y et al [15]	NA	NA	NA	NA	NA	↑ (Apoptotic activity not quantified)	NA	↓	Decreased fold density in *PTEN* organoids
Qian et al [16]	↑	NA	NA	↓	NA	↑	↓	↓	NA
Sacramento et al [17]	↑	NA	NA	NA	NA	NA	NA	NA	NA
Watanabe et al [18]	NA	↓	↓	NA	↓	↑	NA	↓	Increased cell death as measured by propidium iodide
Wells et al [19]	NA	NA	NA	NA	NA	↑	NA	↓	NA
Xu et al [20]	NA	NA	NA	NA	NA	↑	NA	NA	NA
Yoon et al [21]	NA	↓	NA	↓	NA	NA	↓	NA	Decreased percentage of PKC-lambda–positive cells in the ventricular surface Increased percentage of PAX6-positive cells with multipolar morphology
Zhou et al [22]	↑	NA	NA	↓	NA	↑	NA	↓	SOX2-positive cells and TUJ1-positive cells are intermixed and lack discernible organization

Abbreviations: EdU, 5′-ethynyl-2′-deoxyuridine; CASP3, caspase-3; NA, not available; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ZIKV, Zika virus.

Watanabe et al [18] recorded substantially higher values (+20% for both, $P < .001$ unpaired 2-tailed t test and $P < .01$ paired t test, respectively). Qian et al [16] revealed proportionally higher apoptotic activity to ZIKV concentration (+800% at 0.25 dilution, +17 000% at 1.0 dilution, $P < .0005$ t test vs mock-infected controls for both). Similarly, Wells et al [19] showed a positive correlation between CASP3 activity and viral presence ($R^2 = 0.3599$). Finally, Watanabe et al [18] demonstrated elevated CASP3-positive cells in SOX2- (+70%), TBR2- (+65%), and CTIP2-positive cells (+60%) than in mock-infected controls.

Table 4. Cellular Effects of ZIKV According to Lineage

Strains	Viral Replication After 72 h	Preferential Infectivity	Progenitor Cells/ Radial Glia Cells (SOX2+, PAX6+, Nestin+, Phospho-vimentin+)	Intermediate Cells (TBR1+/TBR2+)	Proliferating Cells (Ki67+, Phospho-H3)	Mature Neurons (CTIP2+, TUJ+)	Apoptosis (TUNEL+, CASP3+)	Viability (EdU+)	Change in Size, Area, Volume, or Thickness
African (MR766)	↑ [11,12,16,22]	Progenitor cells > mature neurons [10,16]	↓ [10,11,12,21]	↓ [10]	↓ [10,16,21,22]	↓ [10]	↑ [10,12,16,22]	↓ [16,21]	↓ [11,12,13,15,16,22]
Latin American (PRVABC59, Brazilian, FB-GWUH-2016)	↑ [12,14,17]	Progenitor cells > mature neurons [10,12,18]	↓ [10,12,18]	↓ [10,18]	↓ [10]	↓ [10,12,18]	↑ [10,12,18,19]	NA	↓ [10,12,18,19]
Asian (FSS13025, H/PF/2013)	↑ [12]	Progenitor cells > mature neurons [12,16]	↓ [12]	NA	NA	↓ [12]	↑ [12,20]	NA	↓ [12]

Abbreviations: EdU, 5′-ethynyl-2′-deoxyuridine; CASP3, caspase-3; NA, not available; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ZIKV, Zika virus.

Comparisons of the apoptotic effects according to lineages revealed varying results. The Latin American strain produced greater apoptotic activity than the Africa strain after 24 (+15% and +5%, respectively) and 96 hours postinfection (+30% and +15%, respectively) using TUNEL assay as reported by Cugola et al [10]. However, CASP3 assay attained no difference between strains (+500% for both at 24 hours, +1500% for both at 96 hours). Gabriel et al [12] on the other hand, showed heightened apoptotic activity as early as 2 days postinfection using TUNEL assay for the African strain (+300% vs controls) than the Latin American (+100% vs controls, $P < .001$ ANOVA vs Africa strain) or Asian strains (+60% vs controls, $P < .001$ ANOVA vs African strain). They also found increased TUNEL-positive cells in areas distinct from the ventricular zone for the African strain (+20%, $P < .001$ 2-way ANOVA), as opposed to strains from Latin America or Asia.

Organoid Size

Nine articles reported smaller size, area, volume, or thickness in ZIKV-infected organoids relative to mock-infected controls [10–13, 15, 16, 18, 19, 22]. A 75% decrease in area was reported by both Zhou et al ($P < .001$ unpaired 2-tailed t test) [22] and Li Y et al ($P < .001$ t test) [15], while Garcez et al [13] found a 40% reduction ($P < .05$ unpaired 2-tailed t test). Watanabe et al [18] measured both smaller area (−14%, $P < .001$ paired t test) and perimeter (−20%, $P < .01$ paired t test). Similarly, Dang et al [11] showed diminished volume that was half that of mock-infected controls ($P < .05$ t test).

By comparing ZIKV-infected organoids of different ages at time of infection, Qian et al [16] measured greater reduction in size in younger (−83%, infected at 14 days old, $P < .0005$ t test vs mock-infected controls) than older organoids (−67%, infected at 28 days old, $P < .0005$ t test vs mock-infected controls).

An analysis of lineages by Gabriel et al [12] showed greater reduction in organoid diameter by the African strain (−66%) than either the Latin American or Asian strains (−33% for both) in comparison to mock-infected controls after 11 days of infection ($P < .001$ 1-way ANOVA). Qian et al [16] discovered the level of destruction was proportional to the infectivity of the African strain, leading to a diminished neuronal layer (−50% at 0.25 dilution, $P < .005$ t test; −67% at 1.0 dilution, $P < .0005$) and ventricular zone (−33% at 0.25 dilution, $P < .05$ t test; −50% at 1.0 dilution, $P < .0005$ t test) at increasing viral concentrations. Similarly, Wells et al [19] showed a decrease in area in response to increased MOI for the Latin American strain.

Qian et al [16] observed an enlarged lumen (+200%, $P < .05$ t test) with a reduction in ventricular zone area (−75%, $P < .005$ t test) and thickness (−67%, $P < .0005$ t test) by the African strain in comparison to mock-infected controls. Gabriel et al [12] reported a greater reduction of ventricular zone thickness by the African strain (−100%, $P < .05$ 1-way ANOVA) than Latin American or Asian strains (−57% and −47% respectively,

$P < .001$ 1-way ANOVA for both). Furthermore, they measured a 43% decrease in cortical plate thickness for the Asian strain ($P < .001$ 1-way ANOVA), while Cugola et al [10] showed a 67% and 60% reduction in cortical plate thickness by the Latin American strain, as measured by TBR1- and CTIP2-positive cells respectively ($P < .05$ ANOVA for both).

Risk of Bias Across Studies

MOI was not standardized during the infection process, ranging from under 1 [10, 14, 16, 18–20, 22] to as high as 10 [17, 19]. Furthermore, duration of infection varied greatly from 2 hours [13, 14, 17–19] to more than 24 hours [10–12, 15, 16, 20]. Finally, ages for organoids upon infection varied greatly as there was no agreed standard for organoid maturity.

DISCUSSION

All ZIKV lineages had similar effects on human brain organoids. Currently, only strains from Brazil, Puerto Rico, and French Polynesia, which descended from Asian ancestry, cause microcephaly in human fetuses [23]. Neither strains from Asia nor Africa are known to cause neurological abnormalities [24]. It may be possible that serious neurological consequences are present without phenotypically displaying microcephaly, as recently shown in fetal brains of pigtail macaques [25].

Alternatively, genetic examination of ZIKV reveals evolutionary differences in nucleotides immediately upstream to the Musashi binding element in Latin American strains relative to those from Asia or Africa [26]. This sequence is found in the 3′ untranslated region, which controls gene expression posttranscriptionally. Musashi-1, a neural RNA-binding protein, normally regulates growth and differentiation of neural progenitor cells. Its mutation is commonly found in individuals with autosomal recessive primary microcephaly [27]. As ZIKV upsets neural stem cell function by disrupting Musashi-1 binding to its endogenous targets [28], selective expression of Musashi-1 may explain the variable effects among ZIKV strains.

Consequently, in vitro studies comparing neural progenitor cell mortality found that Latin American and French Polynesian strains induced stronger interferon response and P53 activation, a regulator of apoptosis, than the African strain [29]. This review revealed differences in apoptotic severity among ZIKV lineages. While Cugola et al [10] reported a higher percentage of apoptotic cells caused by the Latin American strain than the African strain, Gabriel et al [12] showed greater apoptosis induced by the African strain than strains from Asia or Latin America. Given the strains are 88.8% nucleic acid /97% amino acid identical [30], further insight into the molecular determinants of the disease warrants investigation.

This is the first systematic review to study the effects of ZIKV on human brain organoids. Overall outcomes are comparable to ZIKV research using 2-dimensional cultures and animal models, like microcephaly, persistent infection, susceptibility of neural progenitors, reduced proliferation, increased neural stem cell death, and mitotic and cell cycle dysregulation [9, 31–36]. Apoptosis was observed both in noninfected cells and in areas distinct from the ventricular zone, perhaps promulgated via a paracrine fashion [37].

More importantly, results achieved with organoids are similar to findings exhibited in fetuses and neonates with congenital ZIKV infection. For example, the enlarged lumen and decreased ventricular zone resemble ventriculomegaly and hydrocephalus [38, 39], the decreased viable cells in the ventricular zone is analogous to periventricular or subventricular calcifications [40–42], while the widespread apoptotic activity and depletion of neuronal precursors were also present in fetal autopsies [43, 44]. ZIKV RNA can be found late in pregnancy [45], which is reflective of persistent viral replication, while the decreasing vulnerability of older organoids supports evidence that earlier infection during gestation causes greater neurological damage to the fetus [46].

Studies selected for this review are constrained by the novelty of the experimental model. Organoid production is expensive and time consuming, limited by variability among batches and lines, irregularities in neuronal maturation, and undesirable cell differentiation into other tissue types [16, 18]. Inevitably, sample sizes are small, which influence the power of statistical tests. It is hoped that cost and reproducibility will be resolved over time.

The greatest limitation among all studies is the lack of standardized protocol governing the experimental process of ZIKV infection of human brain organoids. Thus, this review will provide evidence-based recommendations that cover organoid age, infection duration, and MOI.

Age

Transcriptional profiles conducted by 2 studies revealed that organoid age is proportional to fetal gestational age [16, 18], leading to the recommendation that future experiments use organoids aged 1–5 weeks to represent early-first trimester in humans, and organoids aged 11–14 weeks to represent the mid-second trimester.

Duration of Infection

Only 1 study methodically demonstrated that infection duration influences apoptotic cell death per cortical region [12]. Therefore, 24–48 hours infection is sufficient to measure non-region-specific apoptosis, while 11 days infection is recommended for region-specific differences.

MOI

Three articles achieved ZIKV-induced effects using similarly low ratios of viral particles to infected cells [10, 19, 22]. Thus, investigations into the infection of progenitor cells, apoptotic levels, or change in organoid size, may use an MOI as low as 0.1, with exposure time between 2 and 24 hours.

In summary, this systematic review revealed that ZIKV caused productive replication after 72 hours, preferentially infected neural progenitor cells over mature neurons, reduced

both cell populations, and caused premature differentiation. Limited data involving only African and Latin American strains showed a reduction in populations of proliferating cells and intermediate cells and overall decreased viability. Furthermore, all 3 lineages caused heightened apoptosis and reduction in organoid size. Given the rapid pace of both ZIKV research and organoid improvements, the future holds great promise for a therapeutic solution.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. The author is grateful for the wonderful support and encouragement from Saba University School of Medicine.

Financial support. This work was supported by Saba University School of Medicine.

Potential conflicts of interest. The author reports no potential conflicts of interest. The author has submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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