Ocular Histopathologic Features of Congenital Zika Syndrome

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**IMPORTANCE** Congenital Zika syndrome (CZS) is known to be associated with severe malformations in newborns. Although microcephaly is the hallmark of this disease, the ocular findings are important given the severe visual impairment that has been observed in these patients. Regardless of the increased number of CZS cases reported, to date, no studies have described the ocular histopathologic findings of this entity.

**OBJECTIVES** To evaluate the presence of Zika virus (ZIKV) antigens and describe the associated ocular histopathologic features of 4 cases of CZS.

**DESIGN, SETTING, AND PARTICIPANTS** In this observational case series performed from June 19, 2015, through April 30, 2017, ocular tissue samples from 4 deceased fetuses with a diagnosis of CZS from the National Institute of Health in Colombia were sent to the Florida Lions Ocular Pathology Laboratory for evaluation.

**MAIN OUTCOMES AND MEASURES** The microscopic features of each specimen were described, and immunostaining was performed using a ZIKV NS2B protein antibody.

**RESULTS** Ocular tissue samples from the 4 deceased fetuses (2 female, 2 male) ranging from 21.5 to 29 weeks' gestation with a diagnosis of CZS were studied. The 4 eyes manifested with pupillary membranes, immature anterior chamber angles, loss of pigment and thinning of the retinal pigment epithelium, choroidal thinning, undifferentiated nuclear layers of the retina, and a perivascular inflammatory infiltrate within the choroid. The optic nerve, present in 2 of the eyes, demonstrated atrophy. Expression of ZIKV antigen was present in the iris in cases 1, 3, and 4; the neural retina and choroid in case 1; and in the optic nerve in case 4.

**CONCLUSIONS AND RELEVANCE** Loss of retinal pigment epithelium, the presence of a thin choroid, a perivascular choroidal inflammatory infiltrate, and atrophic changes within the optic nerve were consistently present. These findings may be attributed to ZIKV infection and warrant further study.

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coherence tomography. To our knowledge, no studies have described the ophthalmic histopathologic features of CZS associated with the identification of ZIKV in fetal eyes. We present 4 cases of CZS with viral antigens within cells in the iris, choroid, retina, and optic nerve.

Methods

Sample Collection

In this observational case series, 4 formalin-fixed paraffin-embedded eyes from 4 fetuses diagnosed with CZS from June 19, 2015, through April 30, 2017, by the National Institute of Health in Bogota, Colombia, were submitted to the Florida Lions Eye Bank Ocular Pathology Laboratory at the Bascom Palmer Eye Institute, Miami, Florida, for diagnostic consultation. Initial CZS diagnosis was made at the National Institutes of Health through real-time reverse transcription polymerase chain reaction (RT-PCR) or immunohistochemical analysis using probes and antibodies designed by the Centers of Disease Control and Prevention. Medical records and autopsy reports were collected when available. All samples and associated medical and autopsy records were provided in the context of diagnostic consultation. Institutional review board and ethics committee approval from the University of Miami, Miller School of Medicine, was obtained for this study, and patient consent was waived.

Histopathologic Analysis

In addition to the hematoxylin-eosin and periodic acid-Schiff stains, immunofluorescence and immunohistochemistry stains were performed using a rabbit anti-ZIKV NS2B protein antibody and CD45 (leukocyte common antigen) with red chromogen antibody, respectively. The specimens were compared with a control case (unremarkable eye from a 27-week-old deceased fetus) that was immunostained using the same protocol.

Immunohistochemical Analysis

Immunohistochemical analysis was performed using the standard avidin-biotin-complex technique with an antibody against CD45 (leukocyte common antigen) with red chromogen (Biocare Medical). Grading of the staining intensity was performed by one of us (S.R.D.). The intensity of the immunostaining was graded as negative, weak, moderate, or strong.

Immunofluorescence Analysis

Section slides 4 μm thick were deparaffinized and rehydrated with xylene and washed with a graded series of ethanol and phosphate-buffered saline. Antigen retrieval was performed with Trilogy solution. Blocking was performed with Background Terminator (Biocare Medical). Rabbit anti-Zika virus NS2B protein antibody (GeneTex GTX133308) was used as the primary antibody, diluted in 0.5% Triton and phosphate-buffered saline, and incubated overnight at 4°C. The sections were incubated with goat antihuman Alexa Fluor 488 as the secondary antibody. Slides were mounted in Vectashield (Vector Laboratories) and sealed with clear nail varnish. Sections were visualized with a scanning confocal microscope (Leica). Negative control assays were performed by omission of the primary antibodies. Positive control assays were performed on ZIKV-positive brain tissue samples.

Results

In this case series, ocular tissues of 4 deceased fetuses (2 female, 2 male) ranging from 21.5 to 29 weeks’ gestation with a diagnosis of CZS were studied. The microscopic features of each specimen were described, and immunostaining was performed using a ZIKV NS2B protein antibody.

Case 1

A pregnant woman in her late 30s presented in her second trimester for ultrasonography, which revealed a female fetus with frontal lobe hypoplasia, microcephaly (head circumference, 198.5 mm), craniofusostosis, and microcalcifications located within the cerebellum, cerebral cortex, and retina. Because of the severity of the fetal malformations, the mother underwent voluntary termination of pregnancy. Microscopic examination of cerebral cortex tissue fragments disclosed prominent apoptotic changes with the presence of areas of microcalcification. The results of RT-PCR for ZIKV were positive in the cerebral cortex, brainstem, and spinal cord. The results of immunohistochemical analysis for ZIKV were positive in the cerebral cortex, brainstem, and basal ganglia. One of the eyes was sent for evaluation.

Histopathologic Analysis

Histologic examination disclosed an eye with a moderate amount of autolysis. The corneal endothelium was variably attenuated (autolysis). The anterior chamber angle was open but not fully developed. The iris and crystalline lens were intact. Remnants of a pupillary membrane were present (Figure 1A). The peripheral retina was mostly attached with undifferentiation of the nuclear layers (Figure 1C). The retina in the posterior pole was artifactiously detached (Figure 1D). The attached portions of retina disclosed a thin photoreceptor layer.
Figure 1. Histopathologic Features and Zika Virus (ZIKV) Immunolocalization (Case 1)

A-E, Hematoxylin-eosin (HE)–stained sections of the iris show a pupillary membrane (arrowhead) (A), the posterior retinal pigment epithelium (RPE) displaying foci of thinning with loss of pigment (arrowheads) (B), peripheral retina displaying undifferentiated nuclear layers (asterisk) and a thin photoreceptor layer and unremarkable RPE (arrowhead) (C), posterior pole retina displaying differentiation of the nuclear layers (asterisk) (D), and perivascular lymphoplasmacytic infiltrate within the choroid (arrowheads) (E).

F-H, CD45 (leukocyte common antigen [LCA])–stained sections show the cells within the iris displaying mild to moderate staining (F), cells in the retina displaying mild staining (asterisk) (G), and some cells within the choroid displaying moderate to strong staining (arrowheads) (G and H).

I-K, ZIKV immunolocalization shows the iris displaying positive immunofluorescence staining within the iris sphincter (merged image, arrowheads) (I), fragmented retina displaying positive staining (arrowhead) (J), and choroid displaying positive staining (arrowhead) (K). Original magnification ×100 (A, F, and I), ×200 (C, D, G, H, and J), and ×400 (B, E, L, and K).
The retinal pigment epithelium (RPE) displayed foci of a thinning with relative loss of pigment within the posterior pole compared with that present in the periphery (Figure 1B and C). The mean choroidal thickness was 65 μm. A lymphoplasmacytic infiltrate was present within the choroid-surrounding blood vessels (Figure 1E). A tangentially sectioned portion of optic nerve demonstrated focal atrophy and autolysis. A lymphoplasmacytic infiltrate surrounded an extraocular blood vessel located posteriorly (Figure 2).

**Immunohistochemical Analysis**
Mild CD45 staining highlighting leukocytes was found within the iris (mild to moderate) (Figure 1F), ciliary muscle, and retina (Figure 1G). Moderate staining was observed within the optic nerve. Moderate to strong staining was present within the choroid (Figure 1G and H).

**Immunofluorescence Analysis**
Positive staining for ZIKV was present in the iris sphincter; a few cells were present within the neural retina and choroid (Figure 1I and K), and a few cells surrounding an extraocular blood vessel near a portion of extraocular muscle (Figure 2). No staining was identified in the available sections of cornea, ciliary body, pupillary membrane, RPE, or optic nerve.

**Case 2**
A pregnant woman in her late teens presented in her second trimester for ultrasonography, which revealed anencephaly and absence of brain tissue within the amniotic fluid in the fetus. Because of the severity of the fetal malformations, the mother underwent a voluntary termination of pregnancy. The mother tested negative for human immunodeficiency virus, hepatitis B, syphilis (fluorescent treponemal antibody absorption), toxoplasmosis, and rubella. Microscopic examination of the few brain tissue fragments available disclosed neuroblast apoptosis with ischemic changes. Cerebral tissue and serum RT-PCR results were positive for ZIKV and negative for dengue fever virus and chikungunya virus. The results of cerebral cortex tissue immunohistochemical analysis for ZIKV were negative. One of the eyes was sent for evaluation.

**Histopathologic Analysis**
Histologic examination disclosed an eye with a mild amount of autolysis. The anterior chamber angle was open but not fully developed. The iris and ciliary body were intact. Remnants of a pupillary membrane were present. The retina was artificiably detached. The retinal nuclear layers were undifferentiated in the periphery. The retina within the posterior pole disclosed a thin photoreceptor layer. The RPE had foci of thinning with relative loss of pigment in the posterior pole compared with that present in the periphery. The mean choroidal thickness was 72 μm. A lymphoplasmacytic infiltrate was present within the choroid. No optic nerve was present in the available sections.

**Immunohistochemical Analysis**
Mild CD45 staining highlighting leukocytes was found within the retina. Moderate staining was present within the choroid and ciliary body. Strong staining was present multifocally within the iris and the pupillary membrane. No staining was observed in the corneal epithelium or endothelium.

**Immunofluorescence Analysis**
No staining for ZIKV was identified in the available sections of the specimen.

**Case 3**
A pregnant woman in her early 20s presented in her second trimester for ultrasonography, which revealed a fetus with holoprosencephaly, absence of the corpus callosum, and falx cerebri. Because of the severity of the fetal malformations, the mother underwent a voluntary termination of pregnancy. Microscopic examination of brain tissue disclosed mild apoptotic changes. Fragments of the spinal cord displayed some degenerative changes and reactive gliosis. No inflammation or calcification was identified. The results of brain tissue RT-PCR were positive for ZIKV and negative for dengue fever virus and chikungunya vi...
The results of brain tissue immunohistochemical testing for ZIKV were negative. One of the eyes was sent for evaluation.

### Histopathologic Analysis

Histologic examination disclosed an eye with a marked amount of autolysis. The anterior chamber angle was open but not fully developed. The iris was intact with a pupillary membrane present. The crystalline lens appeared to be intact, with the tunica vasculosa lentis present surrounding the lens capsule. The retina was artifactually detached and fragmented. Some fragments of the peripheral retina appeared to be undifferentiated. The posterior pole retina appeared to be well developed. A thin photoreceptor layer was present. The RPE was mostly absent and, where present, displayed areas of thinning and loss of pigmentation within the posterior pole. A mild inflammatory infiltrate was present within the choroid surrounding some blood vessels and subadjacent to the RPE. The mean choroidal thickness was 82 μm. No optic nerve was identified in the available sections.

### Immunohistochemical Analysis

The results of the CD45 staining highlighting leukocytes were mildly positive within the iris, retina, ciliary body, and choroid.

### Immunofluorescence Analysis

Positive staining for ZIKV was present in the iris sphincter. No staining was identified in the cornea, ciliary body, pupillary membrane, retina, RPE, or choroid.

### Case 4

A pregnant woman in her early 20s presented in her second trimester for routine ultrasonography; imaging revealed a fetus with lissencephaly, ventriculomegaly, microcephaly (head circumference, 230 mm), arthrogryposis, severe retrogastria, and polyhydramnios. Because of the severity of the fetal malformations, the mother underwent a voluntary termination of pregnancy. Microscopic examination of fragments of central nervous system tissue disclosed partial apoptosis of neuroblasts and reactive microglial cells, with no calcifications. The results of brain tissue RT-PCR were positive for ZIKV and negative for herpes simplex virus, dengue fever virus, and chikungunya virus. The results of brain tissue immunohistochemical analysis for ZIKV were negative. One of the eyes was sent for evaluation.

### Histopathologic Analysis

Histologic examination disclosed an eye with a marked amount of autolysis. The corneal endothelium was markedly autolytic. The anterior chamber angle was not fully developed. A thin photoreceptor layer was present. The RPE was present. The retina was artifactually detached, fragmented, and thin. The retina showed differentiation of the nuclear layers in the posterior pole. The retinal nuclear layers were undifferentiated in the periphery. A thin layer of photoreceptors was present in the posterior and peripheral retina. Foci of RPE absence were identified within the posterior pole and periphery. The mean choroidal thickness was 84 μm. A moderate lymphoplasmacytic infiltrate was present within the choroid. Longitudinal sections of the optic nerve disclosed atrophy of the nerve fiber bundles (Figure 3A).

### Immunohistochemical Analysis

Mild CD45 staining highlighting leukocytes was found within the iris, retina, and optic nerve (mild to moderate) (Figure 3B). Strong staining was present multifocally within the choroid.

### Immunofluorescence Analysis

Positive staining for ZIKV was present in the iris sphincter and within a few cells in the optic nerve (Figure 3C). No staining was identified in the cornea, ciliary body, pupillary membrane, retina choroid, or RPE in the available sections.

### Discussion

In the current study, the 4 cases described were confirmed to be congenital ZIKV infection through RT-PCR and/or immunohistochemical analysis of brain tissue. Histopathologic ex-
amination of the 4 eyes demonstrated the presence of a pupillary membrane, an immature anterior chamber angle, lack of differentiation of the nuclear layers of the neural retina, a thin photoreceptor layer, loss of pigment and thinning of the RPE, the presence of a perivascular inflammatory infiltrate within the choroid, and choroidal thinning. The optic nerve, present in 2 of the eyes, demonstrated atrophic change and autolysis. All specimens demonstrated an inflammatory infiltrate predominantly present within the choroid and iris (moderate to strong staining for CD45 with red chromogen). Expression of ZIKV NS2B protein was present in the iris in 3 of the 4 specimens (cases 1, 3, and 4), few cells within the neural retina and choroid in case 1, and cells within the optic nerve in case 4. No ZIKV NS2B protein was identified in the available sections in case 2.

The presence of an immature anterior chamber angle, pupillary membrane, tunica vasculosa lentis, lack of migration of the nuclear layers in the neural retina, and the variable presence of a thin photoreceptor layer are likely to be commensurate with gestational age. The definitive interpretation of other histopathologic findings is more challenging because of the presence of autolysis, fixation artifacts, and mechanical trauma during eye removal. Ciliary body, retinal, and RPE detachment and fragmentation of the neural retina are most likely attributed to autolysis. However, photoreceptor layer thinning, loss of RPE pigment, the presence of a thin choroid, a perivascular choroidal inflammatory infiltrate, and atrophic change within the optic nerve warrant further study in the context of CZV diagnosis.

Several studies have described the spectrum of ocular abnormalities associated with CZS, which more commonly include chorioretinal atrophy, macular pigment mottling, optic nerve pallor, increased cup to disc ratio, and optic nerve hypoplasia with double ring sign. The previously described optical coherence tomographic findings that correlate with some of the aforementioned clinical findings include the discontinuation of the ellipsoid zone and hyperreflectivity underlying the RPE because of atrophy, retinal thinning, choroidal thinning, and colobomatous-like excavation. Discontinuation of the ellipsoid zone, hyperreflectivity underlying the RPE, and choroidal thinning may correlate with the thinning or absence of the photoreceptor layer, loss of pigment and thinning of the RPE, and thinning of the choroid in the 4 cases presented herein.

Although ZIKV is known to affect different fetal organs, the virus has been identified in only fetal human brain tissue, placenta, and retina. Some studies have evaluated ZIKV pathogenesis in the eye in mouse models and human retinal cells. First provided evidence that ZIKV causes retinal lesions by demonstrating that in vitro cultured cells that line the blood-retinal barrier, the retinal endothelium, and RPE were highly permissive and susceptible to ZIKV replication and ZIKV-induced cell death. In addition, the direct intraocular inoculation of the virus in a mouse model disclosed the presence of chorioretinal atrophy with RPE mottling, which are common ocular manifestations of CZS in humans. van den Pol et al found that ZIKV displays an early preference for infecting regions of the developing mouse brain related to vision. These findings suggest that visual impairment in CZS may be attributed not only to retinal infection and subsequent injury but also to infection of the optic nerve and visual cortex.

The expression of ZIKV NS2B protein was observed within cells in the neural retina, choroid, and surrounding an extraocular blood vessel in case 1 and in the optic nerve in case 4, results that correlate with those from the aforementioned studies. These cases also demonstrated choroidal thinning and inflammation and atrophic changes within the RPE and optic nerve. These changes may be attributed to ZIKV infection in the context of CZS diagnosis with the identification of viral antigens in these structures. Although less likely, these findings may be secondary to autolysis.

The 4 eyes disclosed the presence of perivascular inflammatory infiltrates within the choroid. The potential cause of this finding includes choroidal extramedullary hematopoiesis, which has been identified in histopathologic studies of infant eyes. Although the morphologic appearance of the infiltrate was not consistent with choroidal extramedullary hematopoiesis, further characterization of the inflammatory infiltrates was not possible because of the paucity of tissue. Choroidal lymphoplasmacytic infiltrates in the presence of viral antigen in the tissue, accompanied by other histopathologic changes, correlate with the clinical features in CZS. Accordingly, ZIKV infection is the likely cause of these findings.

Of note, ZIKV antigen was present within the iris in 3 of the specimens. The iris pigment epithelium, sphincter, and dilator muscles arise from neuroectoderm. Derivation of muscle from the neuroectoderm is rare and may explain the presence of ZIKV within this muscle and not in the extraocular muscles in the specimens evaluated. Moreover, the presence of virus within cells in the retina, optic nerve, and iris muscles suggests a predilection of ZIKV infection in neuroectodermal-derived structures during development.

The pathophysiologic mechanism in CZV is unclear. On the basis of our findings and previous studies, we hypothesize that the eye represents a target organ for ZIKV during early development through 2 pathways. Zika virus is considered to be a blood-borne pathogen that is transmitted from the mother to the fetus through the placenta. Once in the fetal circulation, the virus may gain access to and infect the RPE and retinal endothelial cells, which constitute the inner and outer blood-retinal barriers, allowing access to the retina. The brain may represent a second pathway through axonal transport into the eye and along the optic nerve. These 2 pathways may facilitate the infection and replication of the virus within different ocular structures and cause damage to the retina and the visual cortex. The presence of choroidal thinning, RPE atrophy, choroidal perivascular lymphoplasmacytic infiltrate, atrophic changes in the optic nerve, and virus within cells in the iris, choroid, retina, optic nerve, and in a perivascular location (case 1) may support this hypothesis.

Limitations
The limitations encountered in this study were related to the small sample size, paucity of tissue, presence of autolysis, and postfixation artifacts. Accurate clinicopathologic correlation was not possible given that gross examination of the 4 specimens,
including gross chorioretinal and optic nerve findings, before paraffin embedding and fixation in formalin was not performed.

Conclusions

Histopathologic studies may contribute to the understanding of the ocular anatomical abnormalities of fetuses and infants with CZS. Although this study is limited by autolysis, lack of precise clinicopathologic correlation, and the quantity of available material, the description of the histopathologic features of CZS and identification of the virus within different human ocular structures add to the understanding of the pathophysiology of this condition. Additional studies are warranted to better characterize the ophthalmic features and mechanisms of CZS in the developing human eye.

REFERENCES