Universidade do Estado do Rio de Janeiro

Centro Biomédico

Faculdade de Ciências Médicas

Kíssila Rabelo

Aspectos imunohistopatológicos e ultraestruturais de placentas de pacientes infectadas por ZIKV na gestação e investigação dos efeitos desta infecção em mastócitos e células trofoblásticas *in vitro*

Rio de Janeiro

2020

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Fisiopatologia Clínica e Experimental, da Universidade do Estado do Rio de Janeiro.

Orientador: Prof. Dr. Jorge José de Carvalho

Coorientador: Prof. Dr. Marciano Viana Paes

Rio de Janeiro

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Assinatura Data

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Aprovada em 09 de julho de 2020.

Banca Examinadora:

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Rio de Janeiro

2020

DEDICATÓRIA

Dedico este trabalho e minha vida aos meus pais, Fátima e Luiz, que são minha base. Nada será capaz de retribuir o amor de vocês. Ao meu amor, Thyago, escolhido por Deus para estar ao meu lado.

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Theodore Roethke

RESUMO

RABELO, Kíssila. *Aspectos imunohistopatológicos e ultraestruturais de placentas de pacientes infectadas por ZIKV na gestação e investigação dos efeitos desta infecção em mastócitos e células trofoblásticas in vitro*. 2020. 210 f. Tese (Doutorado em Fisiopatologia Clínica e Experimental) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

A febre da Zika é uma arbovirose, transmitida principalmente por mosquitos do gênero *Aedes*, mas há também a possibilidade de transmissão vertical, com relatos alarmantes de casos de Síndrome Congênita da Zika associadas à infecção. Sabe-se que o vírus da Zika (ZIKV) atravessa a placenta e infecta o feto, mas a patogênese dessa doença ainda não foi bem esclarecida. A partir disso, o presente estudo visou compreender melhor as consequências que a infecção pelo ZIKV causa no tecido placentário humano e nos tecidos de um natimorto, assim como nas linhagens de células de mastócitos HMC-1 e trofoblásticas HTR-8/SVneo. Os tecidos foram submetidos à análise histopatológica, imunoensaios e hibridização *in situ* para detecção viral, de células imunes e citocinas/mediadores inflamatórios, além da avaliação ultraestrutural das células da placenta. Foram realizados os ensaios de PRNT e RT-PCR tempo real no soro das pacientes. Nas linhagens de células, foi padronizado um protocolo de infecção após ensaio de cinética *in vitro* utilizando imunofluorescência e/ou citometria de fluxo. Em seguida, analisamos as alterações ultraestruturais das células infectadas por microscopia eletrônica de transmissão, observamos a degranulação e liberação de citocinas nos mastócitos ou o estresse oxidativo nas células placentárias. Danos histológicos foram encontrados nas placentas, como a imaturidade vilosa, deposição de fibrina, infiltrado inflamatório, áreas de calcificação e diminuição dos componentes de matriz extracelular. Detectamos células positivas para os antígenos de ZIKV, comprovando inclusive a replicação com a marcação da proteína NS1 e do RNA negativo em diferentes células placentárias e células imunes residentes ou circulantes. O aumento da celularidade (células $CD68^{+}$ e linfócitos $TCD8^{+}$) e da expressão de citocinas pró-inflamatórias locais como IFN-ɣ e TNF-α e outros mediadores, como RANTES/CCL5, MMPs e VEGFR-2, sustentam o caráter da inflamação e disfunção placentária causadas pelo vírus. Além disso, notamos que a diminuição da neurotrofina BDNF pode modular os danos neuronais fetais, já que sua expressão foi menor nas placentas de pacientes que tiveram bebês com microcefalia. Foram observadas também alterações histopatológicas, detecção do vírus e evidências de replicação em todos os órgãos do natimorto que foram analisados. Os aspectos ultraestruturais das células infectadas tanto nos tecidos quanto nas culturas de células apresentaram modificações nas organelas, principalmente no retículo endoplasmático e nas mitocôndrias. Observamos que os mastócitos são permissivos à infecção tanto no tecido placentário quanto *in vitro* e a infecção estimula a degranulação e liberação de citocinas e mediadores. As células trofoblásticas infectadas apresentaram aumento significativo da atividade da enzima antioxidante SOD, das espécies reativas MDA e do ON, enquanto a atividade enzimática de CAT foi menor nas células infectadas pelo ZIKV. Com nossos achados, podemos afirmar que as alterações placentárias causadas pelo ZIKV não são patognomônicas, no entanto, esta infecção leva a alterações placentárias graves, e a infecção algumas vezes resulta no comprometimento ou dano fetal. Os achados apresentados neste trabalho ajudam a entender a imunopatogênese da Zika no contexto materno-fetal.

Palavras-chave: Zika. Placenta. Histopatologia. Ultraestrutura. Linhagem de células HMC-1. Linhagem de células HTR-8/SVneo.

ABSTRACT

RABELO, Kíssila. *Immunohistopathological and ultrastructural aspects of placentae from patients infected with ZIKV during pregnancy and investigation of the effects of this infection on mast and trophoblastic cells in vitro.* 2020. 210 f. Tese (Doutorado em Fisiopatologia Clínica e Experimental) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

Zika fever is an arbovirus, transmitted mainly by mosquitoes of the genus Aedes, but there is also the possibility of vertical transmission, with alarming reports of Congenital Zika Syndrome cases associated with infection. It is known that the Zika virus (ZIKV) crosses the placenta and infects the fetus, but the pathogenesis of this disease has not yet been clarified. From this, the present study aimed to better understand the consequences that ZIKV infection causes on human placental tissue and tissues of a stillbirth, as well as on the HMC-1 mast cell and HTR-8/SVneo trophoblastic cell lines. The tissues were subjected to histopathological analysis, immunoassays and in situ hybridization for viral, immune cells and inflammatory cytokines/mediators detection, in addition to the ultrastructural assessment of placental cells. RT-PCR real-time and PRNT assays were performed with the patient's serum. In cell lines, An infection protocol was standardized after in vitro kinetics assay using immunofluorescence and / or flow cytometry. Then, we analyzed the ultrastructural changes of infected cells by transmission electron microscopy, observed the degranulation and release of cytokines in mast cells or oxidative stress in placental cells. Histological damage was found in the placentae, such as villous immaturity, fibrin deposition, inflammatory infiltrate, areas of calcification and decreased extracellular matrix components. We detected positive cells for the ZIKV antigens, proving even the replication with the NS1 protein and negative RNA labeling in different placental cells and resident or circulating immune cells. The increase in cellularity (CD68+ cells and T CD8+ lymphocytes) and the expression of local proinflammatory cytokines such as IFN-ɣ and TNF-α, and other mediators, such as RANTES/CCL5, MMPs and VEGFR-2, support the character of inflammation and dysfunction placental disease caused by the virus. In addition, we noted that the decrease in BDNF neurotrophin can modulate fetal neuronal damage, since its expression was lower in the placentae of patients who had babies with microcephaly. Histopathological changes, virus detection and evidence of replication were also observed in all stillborn organs analyzed. The ultrastructural aspects of infected cells in both tissues and cell cultures showed changes in the organelles, mainly in the endoplasmic reticulum and mitochondria. We observed that mast cells are permissive to infection in placental tissues and in vitro, and the infection stimulates the degranulation and release of cytokines and mediators. The infected trophoblastic cells showed a significant increase in the activity of the antioxidant enzyme SOD, in the reactive species MDA and NO, while the enzymatic activity of CAT was lower in cells infected by ZIKV. With our findings, we can affirm that the placental changes caused by ZIKV are not pathognomonic, however, this infection leads to severe placental changes, and the infection can results in fetal impairment or damage. The findings presented in this work contribute to understand the immunopathogenesis of Zika in the maternal-fetal context.

Keywords: Zika. Placenta. Histopathology. Ultrastructure. HMC-1 cell line. HTR-8/SVneo cell line.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ADE	*antibody-dependent enhancement*
AIDP	*acute inflammatory demyelinating polyneuropathy*
AMAN	*acute motor axonal neuropathy*
ATP	*adenosine triphosphate*
BDNF	*brain-derived neurotrophic factor*
C	Proteína do Capsídeo
CAT	Catalase
CCL	*chemokine (c-c motif) ligande*
CD	c*luster of differentiation*
CMV	*Human cytomegalovirus*
CPX	*carboxypeptidase X*
CRD	*carbohydrate recognition domain*
CSF	*colony stimulating factor*
CXCL	*chemokine (c-x-c motif) ligande*
DC-SIGN	*dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin*
DMT	Domínio trans-membrana
DNA	*deoxyribonucleic acid*
E	Proteína de Envelope
E. COLI	*Escherichia coli*
EGF	*Endothelial Growth Factor*
FDA	*Food and Drug Administration*
GAGs	Glicosaminoglicanos
GM-CSF	*granulocyte-macrophage colony stimulating factor*
H.E.	Hematoxilina e eosina
hCG	*human chorionic gonadotropin*
hCS	*human chorionic somatomammotropin*
HEV-1	Hepatite E 1
HIV	*Human Immunodeficiency Virus*
HMC	*Human Mast Cell Line*

HSP	*Heat Shock Protein*
HTR	*Human Trophoblast*
iCTB	Células Trofoblásticas Extravilosas Invasivas
IFN	*Interferon*
IFNAR	*Interferon – α/β receptor*
Ig	Imunoglobulina
IGF	*Insulin-like Growth Factor*
IL	*Interleukine*
M	Proteína de Membrana
MAC- ELISA	Teste imunoenzimático de captura de imunoglobulina da classe M
MCP	*Monocyte chemoattractant protein*
MDA	Malondialdeído
MHC	*Major Histocompatibility Complex*
MIA	*maternal immune activation*
MIC	microcefalia
MMP	*Matrix Metalloproteinase*
MOI	*Multiplicity of infection*
MR	*Mannose receptors*
mTOR	*Mammalian Target of Rapamycin*
NADPH	*Nicotinamide Adenine Dinucleotide Phosphate*
NAT	*Nucleic Acid Testing*
NK	*Natural Killer*
NOS	*NO synthases*
NS1	*non structural protein 1*
NS2A	*non structural protein 2A*
NS2B	*non structural protein 2B*
NS3	*non structural protein 3*
NS4A	*non structural protein 4ª*
NS4B	*non structural protein 4B*
NS5	*non structural protein 5*
OMS	Organização Mundial da Saúde
PAS	*Periodic acid-reactive Schiff*
PCNA	*proliferative nuclear cell antigen*

PCR	*Polymerase Chain Reaction*
PGC	*peroxisome proliferator-activated receptor gamma coactivator 1-alpha*
pH	Potencial hidrogeniônico
prM	Proteína pré-membrana
PRNT	*Plaque Reduction Neutralization Test*
PVB19	Parvovirus B19
RE	Retículo Endoplasmático
RNA	*ribonucleic acid*
ROS	*reactive oxygen species*
RT-PCR	*Reverse Transcription Polymerase Chain Reaction*
RT-qPCR	*Quantitative Reverse Transcription Polymerase Chain Reaction*
SCZ	Síndrome Congênita da Zika
SGB	Síndrome de Guillain-Barré
SOD	Superóxido Dismutase
STB	Sinciciotrofoblasto
Sinciciotrofoblasto	SUS
SUS	Sistema Único de Saúde
SV	*Simian Virus*
TGF	*Transforming Growth Factor*
TNF	*Tumor Necrosis Factor*
UCP	*Uncoupling Protein*
UTR	*Untranslated region*
VA	Vilosidade de Ancoragem
VC	Vilosidade Coriônica
vCTB	células citotrofoblásticas vilosas
VEGF	*vascular endothelial growth factor*
VEGFR	*vascular endothelial growth factor receptor*
VHS-1	Vírus da Herpes Simples 1
VHS-2	Vírus da Herpes Simples 2
VZV	*varicella zoster vírus*
ZIKV	vírus Zika

LISTA DE SÍMBOLOS

%	Porcentagem
nm	nanômetro
kb	Kilobases
kDa	Kilodaltons
cm	centímetro
β	Beta
α	Alfa
γ	Gama
Fe^{2+}	Ferro
g	grama
H_2O_2	Peróxido de Hidrogênio
ml	mililitros
OH^-	Radical Hidroxila
ON	Óxido Nítrico

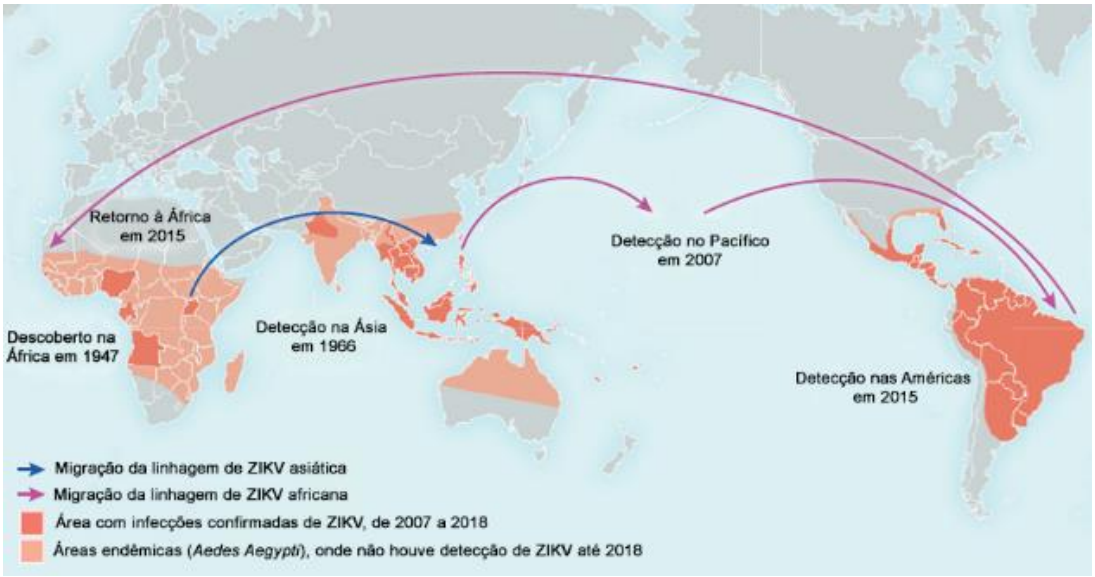
SUMÁRIO

INTRODUÇÃO

A Zika

A febre da Zika é uma importante arbovirose, transmitida principalmente por mosquitos do gênero *Aedes* (subgênero *Stegomyia*), mas também por outras vias incluindo contato sexual, transfusão de sangue e transmissão vertical (1–4). O vírus Zika (ZIKV) foi inicialmente isolado na Uganda em 1947 a partir do sangue de macacos *Rhesus* habitantes da floresta Zika, que deu origem ao nome do vírus (5,6). Desde então, a ocorrência de infecções pelo ZIKV em humanos foi detectada por sorologia de modo esporádico nos continentes africano e asiático. Os países em que ocorreram casos isolados de infecção incluem a Uganda (6), Nigéria (7), Serra Leoa (8), Costa do Marfim (9), Senegal (10), República Centro-Africana (11) e Gabão (12,13) na África; além da Malásia (14), Paquistão (15), Indonésia (16), Micronésia (17,18) e Camboja na Ásia (1,13). Entretanto, as infecções por ZIKV não causaram epidemia até 2007, quando houve uma propagação da doença na Micronésia e em 2013 na Polinésia, se difundindo posteriormente a nível mundial (19–21) (Figura 1).

Figura 1 - Países e territórios com presença corrente ou passada de transmissão de Zika

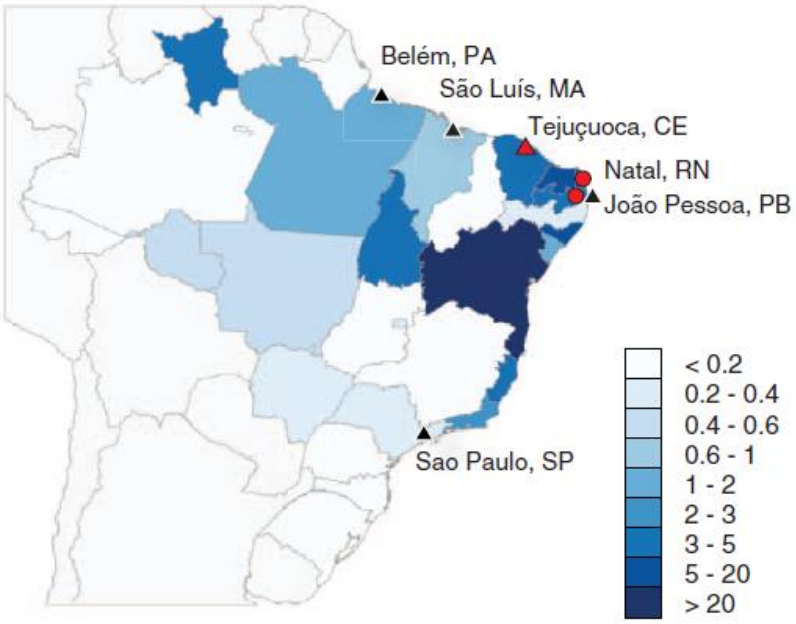


Fonte: Adaptada de Musso et al. (22).

Em 2019, a transmissão autóctone de ZIKV foi confirmada em 87 países ou territórios nas Américas (22). Os grandes surtos ocorreram, claramente, em muitos países e territórios,

devido à introdução do vírus em populações imunologicamente virgens e com presença disseminada dos vetores. No geral, por região, a América do Sul representou 70% dos casos relatados, o Caribe 21%, a América Central 9% e a América do Norte 1%. O maior número de casos suspeitos e confirmados foi relatado no Brasil (346.475 casos, 46%) seguido pela Colômbia (107.206, 14%) e Venezuela (62.200; 8%) (20) (Figura 2). A transmissão do ZIKV diminuiu expressivamente nas Américas desde o final de 2016; menos de 30.000 casos foram relatados em 2018, em comparação com os mais de 500.000 casos reportados no auge da pandemia em 2016 (22).

Figura 2 - Incidência de Zika no Brasil

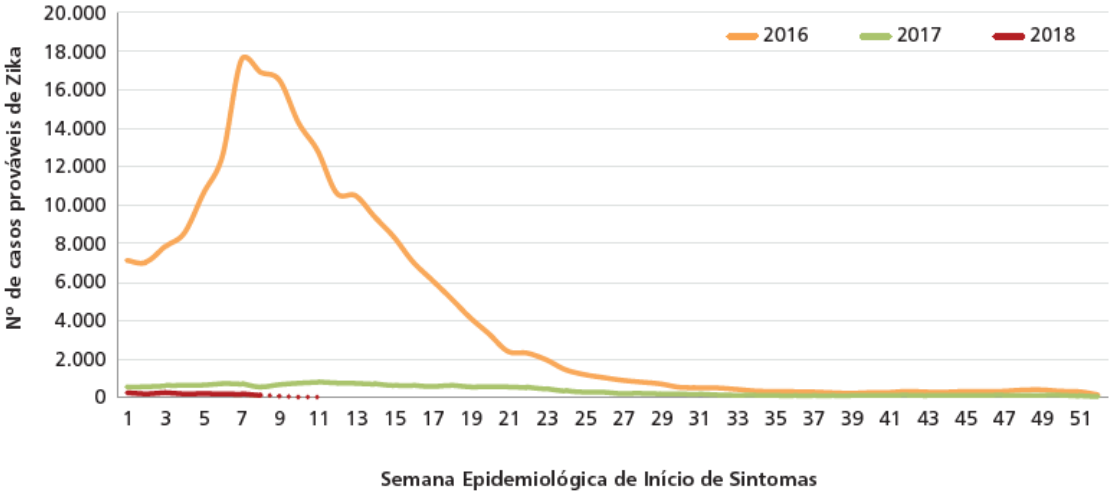


Nota: Incidência total de casos de Zika/100.000 habitantes em cada estado do Brasil em 2015/2016. Círculos e triângulos indicam os diferentes genomas encontrados
Fonte: Adaptada de Faria et al. (23).

No Brasil, a epidemia de Zika foi declarada em 2015 com casos reportados inicialmente na região Nordeste (24), rapidamente propagados por todo o país. Alguns trabalhos com análises filogenéticas retrospectivas sugerem que a introdução do vírus no Brasil pode ter ocorrido em 2013 (20,25,26). A febre do Zika é uma doença de notificação compulsória e, em 2016, foram registrados 216.207 casos no Brasil, com oito óbitos confirmados em laboratório. Dados mais recentes do SUS mostram que houve uma variação no número de casos e óbitos/ano, sendo reportados 17.594 casos prováveis em 2017 com 8 óbitos, 8.680 casos em 2018 com 5 óbitos, 10.768 casos em 2019 com 3 óbitos e até o fevereiro de 2020, 630 casos sem óbitos registrados (27–33) (Figura 3). Ainda sobre o ano de

2020, a Região Norte apresentou o maior número de notificações, com 410 casos. Em seguida, aparecem as regiões Sudeste com 119 casos; Nordeste, 49 casos; Centro-Oeste, 43 casos; e o Sul, com 9 casos (34).

Figura 3 - Número de casos prováveis de Zika no Brasil por semana epidemiológica nos últimos anos



Fonte: Adaptada de Brasil (28).

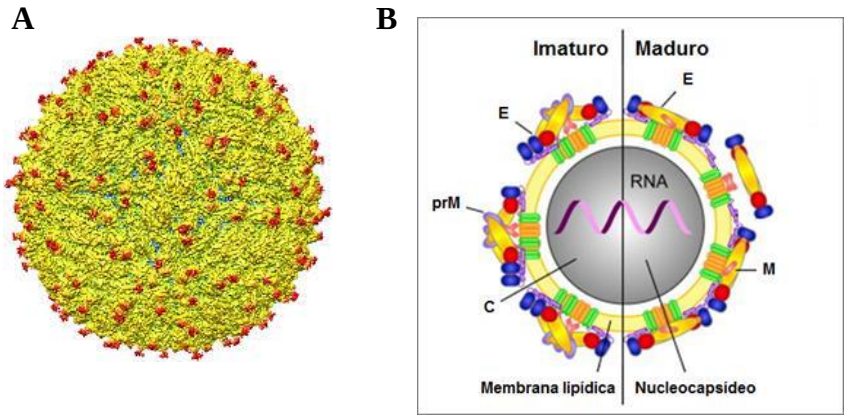
Aspectos virais do Zika

Estrutura e composição bioquímica

Poucos estudos têm relatado a biologia evolutiva do ZIKV (17,35,36), tais estudos descrevem três principais linhagens de ZIKV, um proveniente da Ásia e dois da África. O agente etiológico dessa enfermidade é o vírus Zika, pertencente ao gênero *Flavivírus*, que possui aproximadamente 25-50 nm e compartilha a mesma família (*Flaviviridae*) com outros vírus amplamente conhecidos, como o da dengue, o *West Nile*, o vírus da encefalite japonesa e o da febre-amarela (1,36,37). Os flavivírus são vírus de formato icosaédrico, formados por um envelope composto por uma bicamada lipídica, na qual ficam inseridas as proteínas de envelope (E) e membrana (M). Na porção interna do envelope viral há um nucleocapsídeo, composto por múltiplas moléculas da proteína do capsídeo (C) complexadas ao genoma viral,

uma molécula de ácido ribonucleico (RNA) de fita simples com polaridade positiva (38) (Figura 4).

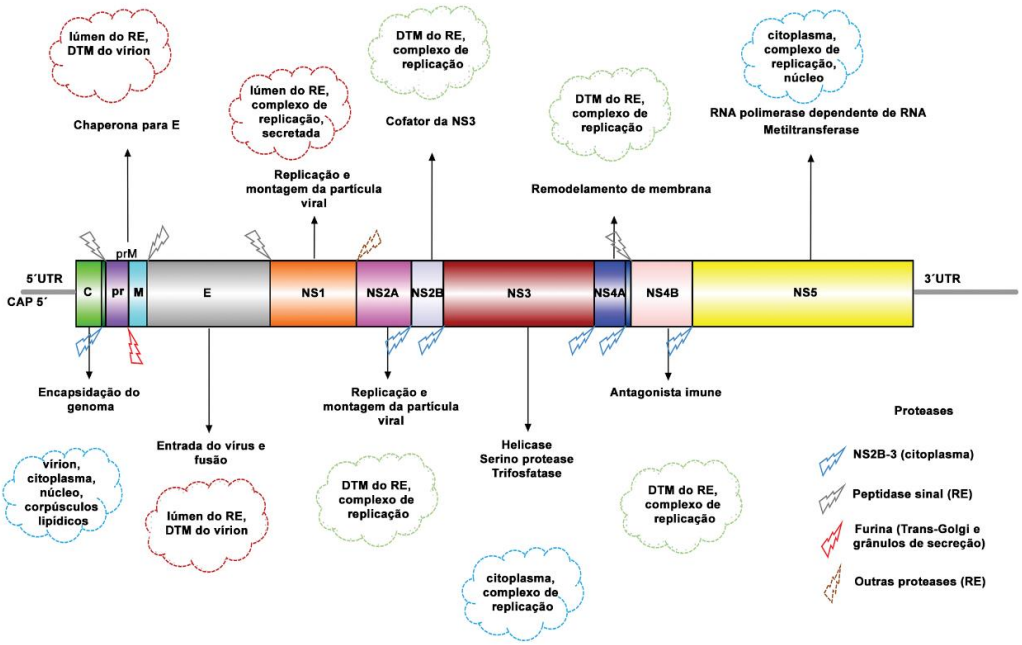
Figura 4 - Representação esquemática do vírus Zika



Legenda: A) Estrutura da partícula viral feita com imagem de microscopia crio-eletrônica; B) A montagem das partículas virais imaturas ocorre no retículo endoplasmático. Antes de sua liberação pela célula, uma protease celular (furina) cliva a proteína pré-membrana (prM) e gera a proteína M, que fica associada à membrana e origina os vírions maduros.
Fonte: Adaptada de Prasad et al. (39) e Stiasny e Heinz (40).

O genoma do vírus tem aproximadamente 11 kb e possui a estrutura *cap* na sua extremidade 5', sendo destituído de cauda poli-A na extremidade 3', e compreende um único quadro de leitura aberto que codifica uma poliproteína precursora das proteínas do flavivírus. Esta poliproteína é inicialmente ancorada no retículo endoplasmático através de hélices transmembranares que atravessam a membrana, sendo subsequentemente clivada por proteases celulares e pela protease viral (proteína NS3/2B), gerando três proteínas estruturais, capsídeo, pré-membrana e envelope, e sete proteínas não estruturais, NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 5). As proteínas C, prM e E são incorporadas às partículas virais durante a sua maturação, enquanto as não estruturais estão envolvidas na replicação e/ou montagem dos vírions. As regiões não-codificantes 3' e 5'(3'UTR e 5'UTR, do inglês *untranslated region*) também são importantes para replicação viral (38,41).

Figura 5 - Genoma e proteínas virais



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Nota: Representação esquemática do RNA viral com as regiões que codificam as três proteínas estruturais C, prM e E, e as sete proteínas não estruturais NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 do vírus Zika, e as regiões não traduzidas 5'UTR e 3'UTR. Os balões mostram a localização mais comum das proteínas na célula, as setas suas principais funções e os raios os pontos de clivagem pelas proteases celulares e viral, no processamento da poliproteína precursora das proteínas flavivirais. DMT- domínio trans-membrana e RE- retículo endoplasmático.

Fonte: Adaptada de Sirohi e Kuhn (42).

Proteínas virais

Entre as proteínas estruturais, a proteína E é o principal constituinte proteico do envelope viral e atua nos eventos de adsorção, possuindo um ou mais sítios de ligação para interagir com o receptor da célula alvo, e na fusão entre o envelope viral e a membrana celular. Esta glicoproteína tem massa molecular de cerca de 53 kDa. A glicoproteína prM tem aproximadamente 26 kDa, e funciona como chaperonina, impedindo que a proteína E sofra mudanças conformacionais durante o seu trânsito por compartimentos ácidos na fase de maturação do vírus. Já a proteína C é altamente básica, com aproximadamente 11 kDa, sendo responsável pela formação do nucleocapsídeo na sua associação ao RNA. Sua porção central contém domínios hidrofóbicos que interagem com membranas celulares, tendo um papel na montagem da partícula viral (1,38,41,43,44).

A primeira das proteínas não estruturais, a glicoproteína não estrutural 1 (NS1) possui cerca de 45-48 kDa e é essencial para viabilidade do ZIKV. Alguns estudos mostram que ela

está envolvida nos estágios iniciais da replicação viral, e no remodelamento do retículo endoplasmático, sendo responsável pelo favorecimento da dilatação de suas cisternas e formação de vesículas (45–47). Foi observado que a proteína NS1 está presente na célula infectada associada à superfície da membrana plasmática. Além disso, essa proteína é secretada para o meio extracelular, sendo encontrada no soro de pacientes durante a fase aguda da infecção (38,41). A proteína transmembranar NS2A possui cerca de 22 kDa e também atua no complexo de replicação, reconhecendo a porção 3' UTR do RNA genômico, além de ser responsável pela evasão imune (48). Já a NS2B (14 kDa) atua como cofator para o domínio protease da NS3, que cliva junções proteicas na poliproteína viral, sendo portanto, essencial para a replicação. A NS3 é uma proteína de aproximadamente 70 kDa, que participa da replicação do vírus (38).

Entre as proteínas não estruturais, a NS3 é a que tem a função melhor elucidada. Ela apresenta múltiplas atividades enzimáticas: a de serino protease, na sua região N-terminal; de helicase, que é dependente de energia; a atividade de Nucleosídeo 5' trifosfatase; além de ser uma RNA trifosfatase 5' terminal, na porção C-terminal (38,44,46,49).

As proteínas NS4A e NS4B são pouco caracterizadas na literatura. A proteína NS4A, com cerca de 16 kDa, aparentemente funciona como âncora da replicase viral à membrana celular e, além disso, possui papel na indução de autofagia nas células infectadas, favorecendo a replicação. A proteína NS4B, com 27 kDa, parece auxiliar na modulação da replicação viral em associação à NS3 (38).

A NS5 é uma proteína com 105 kDa, e que possui diferentes atividades enzimáticas fundamentais à replicação viral. A sua região N-terminal apresenta as atividades guanil-transferase e metil-transferase dependente de S-adenosilmetionina, que são responsáveis pela metilação da extremidade 5' do RNA do vírus, necessária para a formação do *cap* 5´. Já a sua porção C-terminal tem função de RNA polimerase dependente de RNA, que atua na síntese dos RNAs de polaridade negativa (intermediário replicativo) e positiva (38,44,46,48).

Ciclo do vírus

Alguns estudos com flavivírus como o da dengue mostram que existem muitos receptores para a entrada destes vírus em células de mamífero, sendo os candidatos mais comuns os glicosaminoglicanos (GAGs), como o heparan sulfato. Essa ligação ocorre através

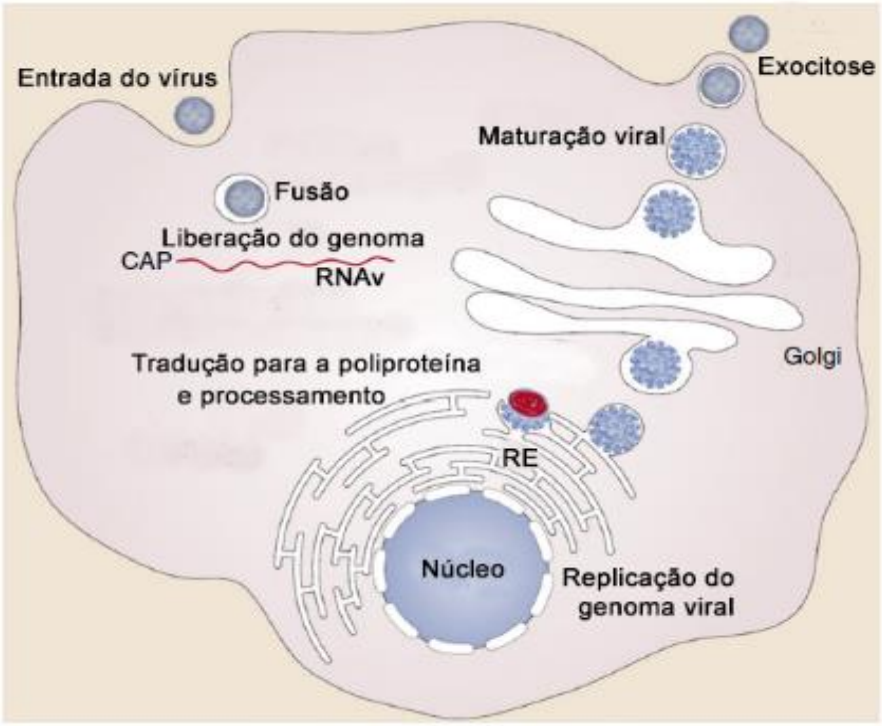
de dois resíduos de lisina (K291 e K295) localizados no domínio III da proteína E do vírus (50). Outro receptor importante, presente principalmente nas células dendríticas, pertence à família das lectinas: *Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin* (DC-SIGN) (51). Esta proteína possui um domínio de reconhecimento de carboidrato (CRD) que se liga a uma porção rica em manose, no resíduo de aminoácido N67 da proteína E (52). Em macrófagos, os receptores de manose (MR) foram observados como principais receptores para o vírus da dengue. Seus CRDs são capazes de reconhecer os glicanos da proteína E dos quatro sorotipos virais, possivelmente nos aminoácidos N67 e/ou N153 (53). Além destes, existem outros receptores importantes já relatados, dentre eles estão: as proteínas de choque térmico 70 e 90 (HSP 70 e 90), TIM/TAM, GRP78/Bip, CD14 e proteína 37/67 receptora de laminina de alta afinidade (54–57).

Alguns trabalhos têm explorado a entrada do ZIKV nas células. O receptor Axl, da família dos receptores tirosina quinase TAM, parece ser um dos mais importantes na entrada do ZIKV em diferentes tipos celulares. Um estudo mostrou que a entrada do vírus em células de Sertoli ocorre pricipalmente através deste receptor, enquanto outros receptores desta mesma família como Tyro-3, Mer e os da família tirosina quinase TIM não afetam a replicação viral de modo significativo (58). Adicionalmente, anticorpos neutralizantes e RNA de interferência para Axl reduziram drasticamente a infecção por ZIKV em fibroblastos dermais (59). De acordo com este modelo, os estudos confirmaram que as células progenitoras neurais humanas, que são permissivas a infecção por ZIKV tanto *in vitro* como *in vivo* expressam Axl (60–62). O RNAm de Axl está presente também em outras células cerebrais, incluindo células gliais radiais, astrócitos e células microgliais (63–65). Adicionalmente, Tabata et al. (66), realizaram um estudo em células explantadas da placenta, além de linhagens celulares, no qual observaram que muitas das células placentárias (citotrofoblastos, fibroblastos, células deciduais e endoteliais), assim como as células do sistema imune que estavam presentes no tecido - como macrófagos e células dendríticas - possuíam os diferentes receptores de superfície aos quais os flavivírus são capazes de se ligar e mediar sua entrada na célula por endocitose. Esses receptores incluem as proteínas da família tirosina-quinases (TAM) como Tyro3, Axl e Mertk; TIM1, uma imunoglobulina produzida por células T e as proteínas DC-SIGN nos fibroblastos, que permitiram que as diferentes células fossem suscetíveis à infecção pelo ZIKV (66).

Para a entrada, o vírus interage com a célula pela ligação da proteína E com o receptor celular e penetra na célula alvo por meio de um mecanismo de endocitose mediado por clatrina (67–69). Após a endocitose, o pH ácido do endossoma faz com que a proteína E sofra

mudanças conformacionais, expondo seu peptídio de fusão, que resulta na fusão do envelope viral com a membrana da vesícula. Em seguida, o nucleocapsídeo é liberado no citoplasma e o genoma é traduzido na poliproteína precursora das proteínas flavivirais. Posteriormente, ocorre o processamento da poliproteína gerando as proteínas virais. Também são sintetizadas (através da ação da proteína NS5) moléculas de RNA de polaridade negativa, que são os intermediários (molde) para novas moléculas de RNA de polaridade positiva. A montagem da partícula viral e sua maturação, com a clivagem da proteína prM, ocorre no retículo endoplasmático e complexo golgiense, sendo posteriormente exocitada (40,69) (Figura 6).

Figura 6 - Ciclo de replicação do vírus



Nota: Esquema representativo do ciclo de replicação do ZIKV em uma célula suscetível. O ZIKV interage diretamente com receptores presentes na superfície da célula alvo, em seguida, a partícula é internalizada em uma vesícula mediante um mecanismo envolvendo clatrinas, dentro da qual ocorre alteração de pH levando a fusão das membranas viral e celular e a consequente liberação do genoma viral para o citosol. Os processos de replicação do genoma viral e a montagem de novas partículas ocorrem no retículo endoplasmático e posteriormente os vírions formados são direcionados para a rede trans-Golgi. No complexo golgiense a furina, uma protease celular, cliva a proteína prM em M, originando partículas maduras ou parcialmente maduras, as quais são exocitadas, podendo infectar outras células.

Fonte: Adaptada de Green et al.(70).

A placenta

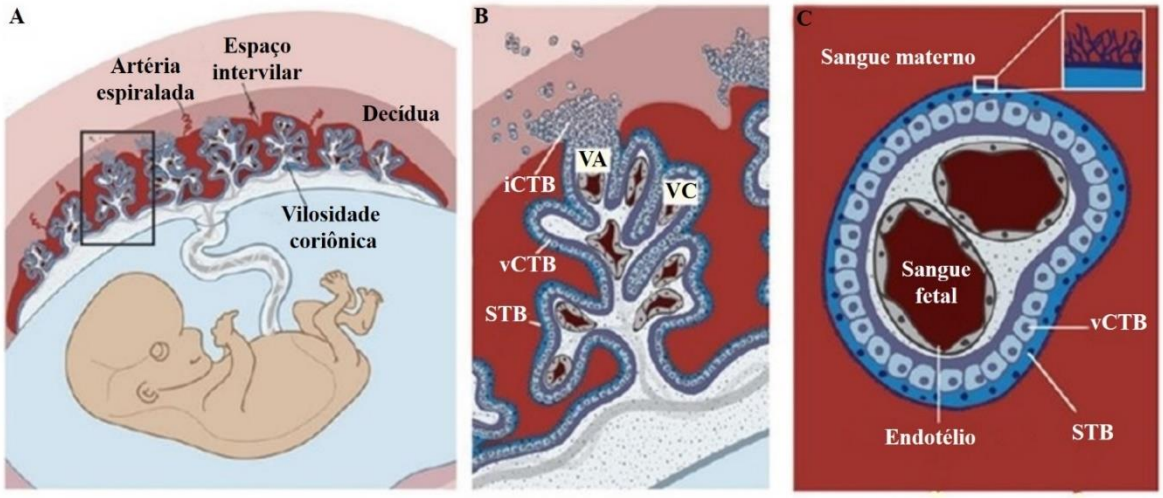
Histologia da placenta

A placenta é caracterizada como um órgão temporário e quimérico, formado durante a gestação a partir de tecido materno e fetal, cujas funções são essenciais para uma gestação saudável. Este órgão é responsável pela nutrição, troca gasosa, remoção de resíduos tóxicos, além de proporcionar um suporte endócrino e imunológico ao feto, regulando a fisiologia da mãe e do feto ao longo da gestação e durante o parto (71). Problemas na formação da placenta podem causar complicações gestacionais que resultam em morbidades e até mortalidade do feto ou da mãe, como pré-eclâmpsia e restrição do crescimento fetal (72–74). A placenta possui uma estrutura bastante complexa formada por tecido materno e diversos tipos de células trofoblásticas derivadas do embrião. As células trofoblásticas são células epiteliais especializadas para diversas funções durante a gestação (71). A placenta humana é do tipo hemocorial, na qual o sangue materno fica em contato com células trofoblásticas de origem fetal.

A placentação hemo-monocoriônica humana é caracterizada por uma invasão fetal única dos trofoblastos na decídua. A porção fetal da placenta é constituída por trofoblastos e mesoderma extraembrionário (placa coriônica) que inicialmente proliferam muito mais rapidamente do que o embrião após o implante (75,76). No segundo mês, o trofoblasto é caracterizado por um grande número de vilosidades coriônicas secundárias e terciárias, com aparência radial. A estrutura funcional da placenta é constituída das vilosidades coriônicas e possui três camadas: a camada de sinciotrofoblastos (multinuclear), que fica na superfície; a camada de células citotrofoblásticas (mononuclear) e o mesoderma com o endotélio dos vasos fetais (que separa o sangue materno do sangue fetal). O sangue materno é liberado na placenta pelas artérias espiraladas no útero e a erosão desses vasos libera o sangue nos espaços intervilosos, que ocorre por invasão endovascular causada pelas células citotrofoblásticas. Estas células, liberadas das vilosidades de ancoragem, invadem as extremidades das artérias espiraladas, onde substituem as células endoteliais maternas e criam vasos híbridos com células maternas e fetais (Figura 7). A partir da oitava semana de gestação, é possível também observar o córion liso, uma camada relativamente avascularizada e o córion

frondoso, que corresponde ao componente fetal da placenta. Posteriormente, o córion frondoso é dividido em áreas denominadas cotilédones (77–80).

Figura 7 – Histologia da placenta

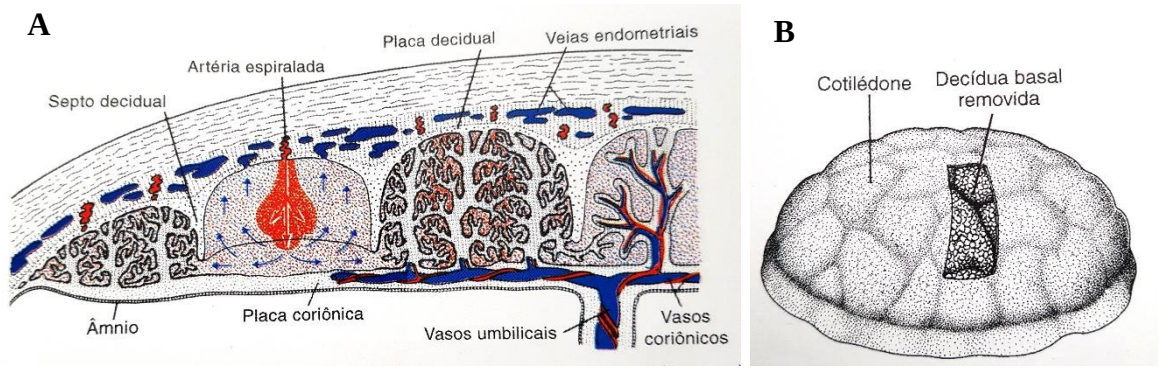


Legenda: A) Células trofoblásticas invadem as artérias espiraladas maternas levando à perfusão dos espaços intervilosos;) Na vilosidade coriônica (VC) células citotrofoblásticas vilosas (vCTB) produzem uma camada multinucleada de sinciciotrofoblasto (STB) que fica em contato com o sangue materno. As células citotrofobásticas que ficam na extremidade das vilosidades e em contato com a parede uterina se diferenciam em células trofoblásticas extravilosas invasivas (iCTB) que invadem a decídua na vilosidade de ancoragem (VA); C) O corte transversal da vilosidade coriônica mostrando a composição da membrana placentária: STB em contato com o sangue materno, contém microvilosidades para elevar a velocidade de troca entre a circulação materna e a fetal, seguida de vCTB, mesênquima e vaso fetal envolvido por endotélio

Fonte: Adaptada de Maltepe et al. (81).

A decídua basal é o componente materno da placenta, formada a partir do endométrio. Os vasos nessa porção do endométrio suprem os espaços intervilares com sangue. Por volta do quarto mês está estabelecida a zona juncional, na qual o trofoblasto (células gigantes sinciciais) e as células deciduais se misturam, sendo também rica em material amorfo. Depois deste período, a maior parte das células trofoblásticas se degenera, restando apenas a placa decidual e a placa coriônica, com os espaços intervilosos. Durante o quarto e quinto meses a decídua forma vários septos deciduais, os quais se projetam nos espaços intervilosos (Figura 8) (77–79).

Figura 8 - A placenta na segunda metade da gravidez

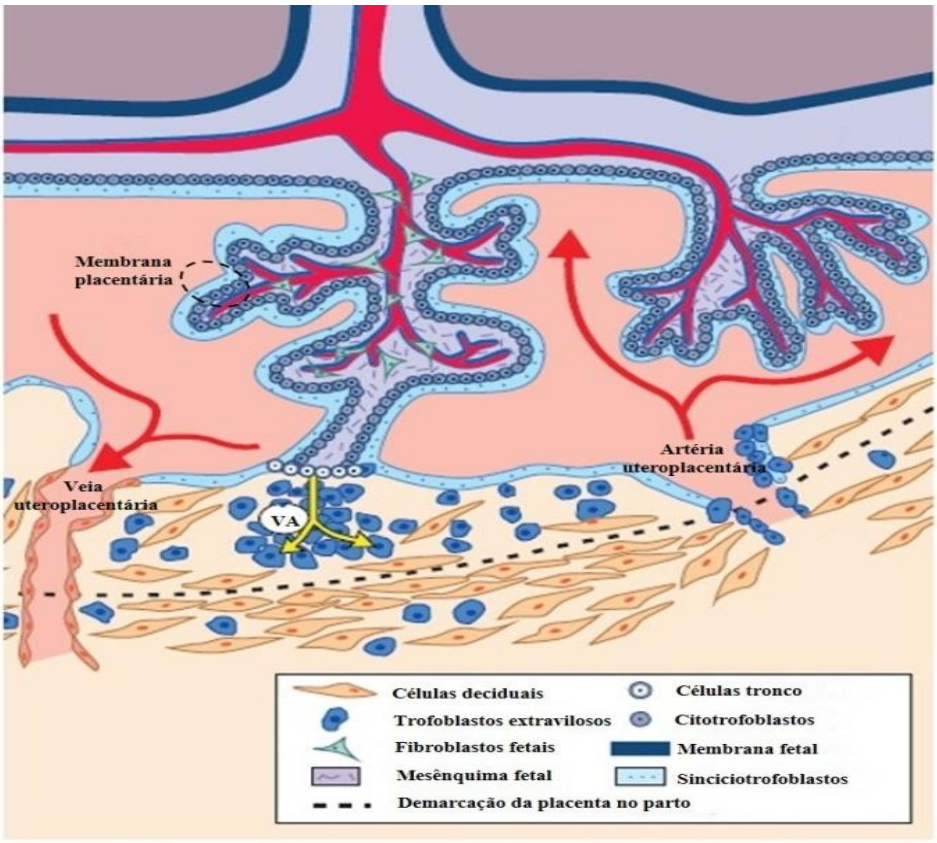


Legenda: (A) Septos deciduais em corte transversal. As setas indicam o retorno do sangue interviloso para a circulação materna. (B) Os cotilédones estão parcialmente separados pelo septo decidual.
Fonte: Adaptada de Sadler (77).

Assim, o sangue materno e o fetal não se misturam, exceto pela ruptura das paredes capilares, que acontece raramente fora da situação de parto. Essa separação entre os sangues fetal e materno denomina-se membrana ou barreira placentária. Ela é garantida por quatro camadas: (1) endotélio fetal, (2) o tecido conjuntivo no eixo da vilosidade com células mesenquimais e fibroblastos, (3) o citotrofoblasto e (4) o sinciciotrofoblasto. Entretanto, com o amadurecimento da placenta, essa camada fica cada vez mais delgada para facilitar a troca de produtos pela membrana placentária, mas compacta o suficiente para evitar muitos tipos de infecções (Figura 9) (77–79).

Com o crescimento do feto e da expansão do útero, a placenta aumenta de tamanho ao longo da gravidez. Ao final da gestação, a placenta reveste de 15 a 30% da superfície interna do útero. O aumento na espessura da placenta também ocorre, e é devido a arborização das vilosidades. Quando chega a termo, a placenta possui forma discoidal, com 15 a 20 cm de diâmetro, aproximadamente 3 cm de espessura e 500 a 600 g de peso. A inserção do cordão umbilical é excêntrica ou até mesmo marginal (77).

Figura 9 - Representação esquemática do corte transversal da placenta mostrando a vilosidade coriônica, a decídua e a membrana placentária



Nota: As células deciduais são grandes morfologicamente, arredondadas e levemente achatadas. A vilosidade coriônica é constituída por células mesenquimais e fibroblastos fetais; citotrofoblastos, com característica epitelial, ou seja, uma camada de células quadradas dispostas lado a lado; estes citotrofoblastos podem se diferenciar e fusionar formando o sinciciotrofoblasto, uma grande camada multinucleada, que pode formar os nós sinciciais; citotrofoblastos se diferenciam também como células-tronco trofoblastos invasivos extravilosos na vilosidade de ancoragem (VA).

Fonte: Adaptada de Novakovic et al. (82).

Além disso, a matriz extracelular é um componente essencial da placenta, pois compreende a sinalização bioquímica e biofísica fornecida às células. A matriz fornece valiosas percepções sobre a situação biológica do órgão, pois a manutenção da homeostase pode ser compreendida com a dinâmica da regulação que ocorre entre a matriz e as proteínas celulares. Proteínas como a laminina, colágeno IV e fibronectina estão presentes na placenta auxiliando a interação entre células, se ligando a fatores de crescimento endotelial e estimuladores de angiogênese (83). Ao final da gravidez, há aumento de tecido fibroso no eixo da vilosidade, espessamento da membrana basal dos capilares fetais e deposição de fibrinóide na superfície das vilosidades. Essa formação excessiva de fibrinóide pode causar infarto de um espaço intervilar (77).

Fisiologia da placenta

As principais funções da placenta são a troca de produtos gasosos e metabólicos entre a corrente sanguínea materna e fetal e a produção de hormônios (77).

O sangue materno chega a placenta através de 80 a 100 artérias endometriais espiraladas que penetram na decídua basal. Cerca de 150 ml de sangue é renovado de três a quatro vezes por minuto, levando a troca de gases e metabólitos. A troca de gases como o oxigênio, o dióxido e o monóxido de carbono ocorrem por difusão simples e qualquer interrupção, mesmo que por pouco tempo, pode ser fatal para o feto. Quando a termo, o feto extrai de 20 a 30 ml de oxigênio por minuto da circulação materna. A troca de nutrientes e eletrólitos (água, aminoácidos, ácidos graxos, carboidratos, hormônios, vitaminas e alguns anticorpos) é rápida, acontece de diferentes formas e aumenta cada vez mais com o avanço da gestação. Há uma grande eliminação de produtos metabólicos inaproveitáveis, água e dióxido de carbono do sangue fetal para o materno. Entretanto, antes mesmo do estabelecimento do fluxo sanguíneo pela placenta, o crescimento do embrião tem suporte a partir dos produtos metabólicos sintetizados pelo trofoblasto ou transportados por ele. O sinciciotrofoblasto sintetiza glicogênio, colesterol e ácidos graxos, além de outros nutrientes (77,84).

A placenta também possui função endócrina, e produz hormônios peptídicos e esteroides. Esses os hormônios são sintetizados no sinciciotrofoblasto e no quarto mês, a placenta já é autossuficiente em progesterona caso o corpo lúteo deixe de funcionar de modo adequado. A placenta também produz estrogênio, principalmente o estriol, de modo progressivo até o final da gravidez, a fim de estimular o crescimento uterino e o desenvolvimento das glândulas mamárias. Contudo, a produção de estrogênios é dependente do feto. Como a placenta é desprovida de enzimas necessárias para a produção dos precursores estrógenos, é estabelecida a cooperação fetoplacentária, na qual os precursores são produzidos no córtex da supra-renal (adrenal) fetal, podendo, inclusive, a produção de estrógenos ser utilizada como um sinalizador do desenvolvimento fetal (77,84).

Os outros hormônios são peptídicos e sintetizados por diferentes células da placenta. Nos dois primeiros meses, a gonadotrofina coriônica humana (hCG, *human Chorionic Gonadotropin*), o primeiro hormônio peptídico, é produzido e excretado no útero, para manter o corpo lúteo, sendo muito utilizado como indicativo no testes de gravidez. Outro hormônio produzido pelo sinciciotrofoblasto é a somatomamotrofina (hCS, *human Chorionic Somatomammotropin*), que além de estimular o desenvolvimento das mamas para a produção

de leite, também prioriza o recebimento de glicose pelo feto, através do sangue materno, exercendo um efeito de estímulo a diabetes na mãe. Os *Insulin-like Growth Factor protein* (IGF-I e o IGF-II) (são produzidos pelo citotrofoblasto e estimulam sua própria proliferação e diferenciação. Já o fator de crescimento endotelial (EGF, *Epidermal Growth Factor*), na placenta de 4 a 5 semanas é sintetizado pelo citotrofoblasto, estimulando a proliferação, enquanto de 6 a 12 semanas é produzido pelo sinciciotrofoblasto, estimulando e mantendo sua função já diferenciada. A relaxina é sintetizada pelas células da decídua, sendo responsável pelo amolecimento do colo e dos ligamentos pélvicos no final da gestação. Já a leptina é produzida pelo sinciciotrofoblasto, principalmente no último mês de gestação, regulando a reserva de nutrientes maternos às necessidades do feto (77,84).

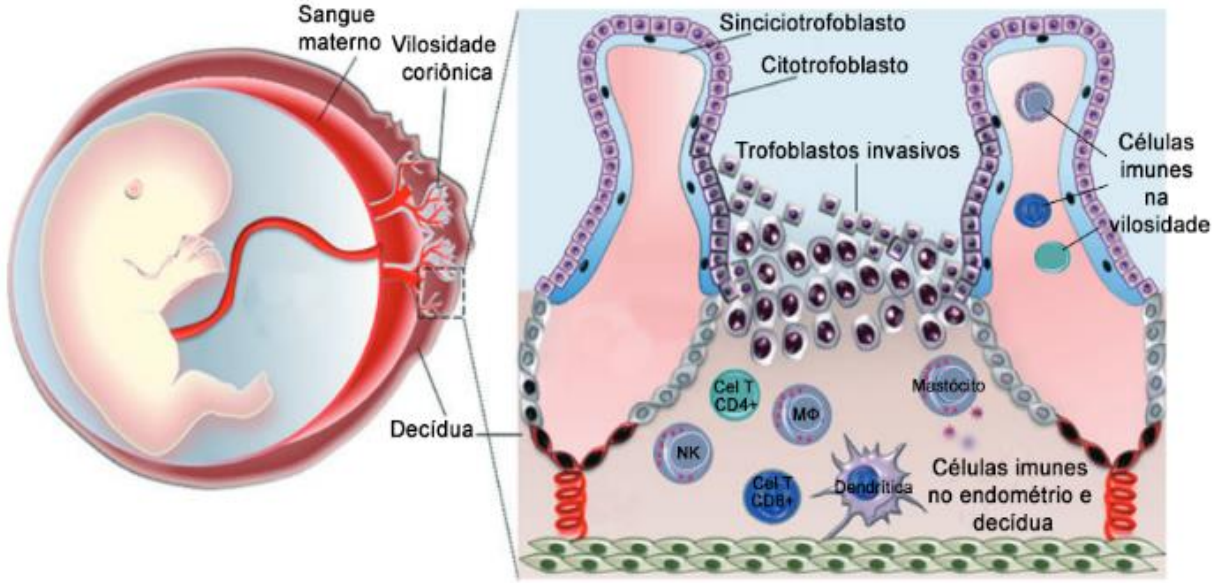
Há ainda outros fatores de crescimento, envolvidos na proliferação do citotrofoblasto como o *Colony Stimulating Factor* (CSF-1), o fator de crescimento fibroblástico, o fator de crescimento derivado de plaquetas e as interleucinas 1 e 3, além do fator de necrose tumoral (TNF, *Tumor Necrosis Factor*) que pode inibir a proliferação trofoblástica (84).

Imunologia da placenta

O estado imunológico da grávida foi durante muito tempo entendido como suprimido, mas trabalhos atuais mostram evidências de que as respostas imunológicas na interface materno-fetal não são simplesmente suprimidas, mas que são altamente dinâmicas (85). A gravidez é um grande paradoxo imunológico, tendo em vista que o feto e a placenta consistem em um enxerto semi-alogênico, que deveria ser automaticamente rejeitado por um hospedeiro imunologicamente competente. No entanto, o feto é protegido contra agressões imunológicas, sugerindo que há adaptações complexas de ambas as partes para que o sistema imune atue em função da tolerância, em vez da rejeição (86).

A placenta humana tem como característica o estabelecimento de interfaces fetais-maternas imunoprivilegiadas, onde os tecidos fetais estão em estreito contato com o sistema imunitário materno (75). Durante a gravidez, a decídua basal contém um rico infiltrado imunológico, que afeta e pode ser afetado de acordo com a dinâmica da interface materno-fetal. As duas principais interfaces são a própria decídua que serve de ponto de ancoragem para a placenta e o espaço interviloso, local onde o sangue materno permeia a vilosidades placentárias flutuantes (75,86) (Figura 10).

Figura 10 - Representação esquemática da interface fetal-materna, com as células imunes que auxiliam a implantação e manutenção da gravidez



Nota: A figura mostra que tanto o endométrio, a decídua materna e as vilosidades coriônicas possuem células imunes que são essenciais para a implantação, manutenção da gravidez e defesa contra possíveis patógenos.
Fonte: Adaptada de Jabrane et. al. (86).

Já foi observado que um ambiente pró-inflamatório é necessário antes mesmo da implantação, e não como o resultado de uma implantação bem-sucedida. Leucócitos deciduais, especialmente células dendríticas e natural killer (NK), auxiliam na aposição, adesão e invasão do blastocisto (86–88). À medida que o blastocisto invade o endométrio e é formada a decídua, as células trofoblásticas aumentam a população de leucócitos deciduais por quimiotaxia, via secreção de citocinas como a CXCL12, CXCL8, TGF-β e CCL2, e induz a diferenciação de células NK, macrófagos e células T CD4+ através de a secreção de IL-15, GM-CSF e TGF-β, respectivamente (89,90).

Aproximadamente 70% dos leucócitos deciduais são células natural killer (NK), 20 a 25% são macrófagos, 1,7% são células dendríticas e aproximadamente 3 a 10% são células T e mastócitos. As células B são encontradas, mas em baixa quantidade (85). Mesmo com essa característica pró-inflamatória, a tolerância materna ao tecido fetal é estabelecida, em parte, pelo comportamento único das células dendríticas deciduais, que não migram para os vasos linfáticos maternos após a exposição ao antígeno fetal, contrário ao que ocorre na apresentação clássica de antígeno pelas células dendríticas normais. O que acontece na gravidez é um evento único, no qual os antígenos fetais chegam sem estarem processados ou ligados a qualquer célula nos nódulos linfáticos através do transporte passivo e são

apresentados a células T por células dendríticas residentes dos linfonodos, um paradigma que não produz respostas imunes significativas (80,91).

Ou seja, o ambiente pró-inflamatório no início da gestação dá suporte a invasão trofoblástica e a remodelação decidual, um processo necessário para criar uma placenta que possa suprir e amparar o feto ao longo da gravidez. No segundo trimestre, o crescimento da placenta diminui, e o ambiente periférico muda e passa a ser anti-inflamatório. Isso permite a transferência máxima de oxigênio para o feto e corresponde ao período de crescimento fetal substancial. As células de Hofbauer (macrófagos residentes da placenta) e as células T reguladoras ajudam a modular o ambiente secretando citocinas antinflamatórias e prevenindo a resposta imune do tipo efetor voltada para o tecido fetal (89,92).

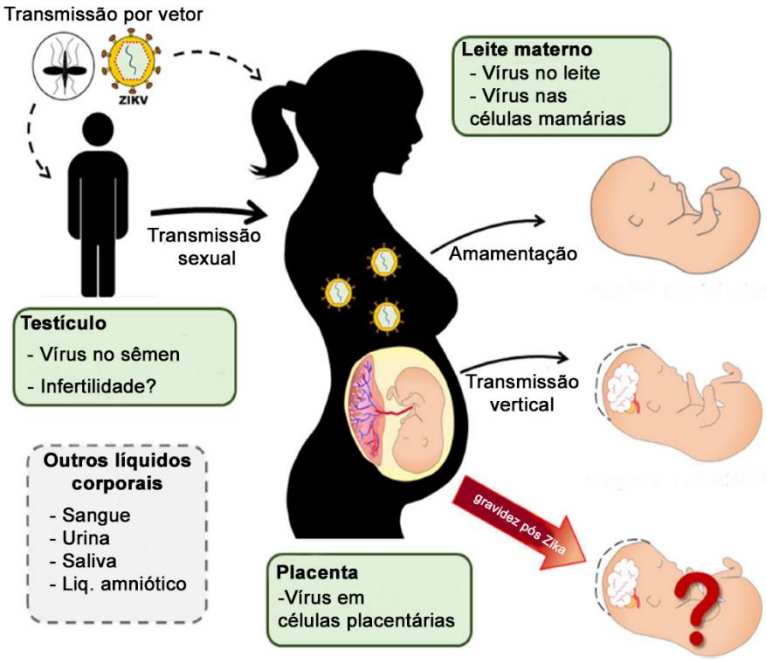
As células de Hofbauer são abundantes em comparação a maior parte dos leucócitos placentários e possuem papel importante na imunologia da placenta, sendo detectados a partir do 10º dia de gestação. Os macrófagos ligam o sistema imune inato e adaptativo por meio da fagocitose, processamento e apresentação de antígenos a linfócitos T. O papel dos macrófagos na orquestração da inflamação, respostas imunes e cicatrização/remodelação tecidual tem sido um campo de estudo intensivo e ainda necessita de maior elucidação da sua atividade na placenta (93).

Além dos macrófagos, os mastócitos são células imunes abundantes na placenta e que possuem papel importante nas reações imunológicas em geral (94,95). Pelo fato de estarem presentes no endométrio e decídua, os mastócitos contribuem funcionalmente para a implantação e seus eventos subsequentes, como a remodelação tecidual e angiogênese pela secreção de mediadores e citocinas, após serem ativados pelo estímulo hormonal (96).

Transmissão do vírus Zika

A transmissão do ZIKV pode ocorrer por diferentes vias além da já amplamente conhecida para outros flavivírus, transmissão por vetor. As possibilidades de transmissão foram sendo descobertas após o grande surto de Zika de 2015, com detecção da transmissão sexual, sanguínea, vertical e até mesmo pelo leite materno (Figura 11). Os trabalhos que discutem essas descobertas serão descritas a seguir.

Figura 11 - Possíveis vias de transmissão de ZIKV



Nota: A transmissão pode ocorrer através do repasto do mosquito vetor, pelo contato sexual com parceiro infectado, via transplacentária ou até mesmo na amamentação. O vírus é encontrado também em outros líquidos corporais, o que pode levar a outros tipos de transmissão. Não se sabe ainda se uma mulher que foi infectada pode transmitir para o feto em uma futura gestação, após o período de infecção.
Fonte: Adaptada de Teixeira et. al. (97).

Transmissão por vetores

O ZIKV é transmitido principalmente por mosquitos do gênero *Aedes*, tendo como vetores mais frequentes o *Aedes aegypti* e *Aedes albopictus*. Estes insetos estão presentes nas regiões tropicais e subtropicais do planeta, e por isso estas são as áreas de maior incidência da doença (1,2). A circulação do ZIKV foi documentada em dois ciclos de transmissão ecológica e evolutivamente distintos: um ciclo silvestre, onde o vírus circula entre os mosquitos e os primatas não humanos; e um ciclo humano ou urbano, entre humanos e peridomicílios/domésticos e mosquitos. Em humanos, após a picada do mosquito, há um período de incubação que pode levar de seis a nove dias, e então os sintomas se seguem (21,98).

Transmissão sexual

Antes mesmo do surto de Zika dos últimos anos, já havia sido relatada a possível transmissão sexual do vírus (3,98). Os pacientes expostos a áreas endêmicas apresentaram sintomas da doença e um sinal antes considerado atípico em infecções por flavivírus, a hematospermia. Nesses casos, a presença de vírus no sêmen foi confirmada por testes sorológicos ou por RT-PCR. Além disso, foi relatado que os parceiros sexuais desses pacientes apresentaram sintomas semelhantes, fortalecendo essa hipótese (98). No Brasil, durante a epidemia de ZIKV, foi observado que a incidência de infecção em mulheres e homens adultos seria maior do que em crianças, o que indica a transmissão sexual. A transmissão sexual é possível a partir de infecções assintomáticas e sintomáticas através de relações sexuais genitais, orais e anais, e de contato masculino para masculino, de masculino para feminino, e de contato feminino para masculino (99). Entretanto, o número de mulheres infectadas é maior que o de homens, sugerindo que a infecção ocorra através do sêmen, já que as mulheres geralmente são mais suscetíveis às doenças sexualmente transmissíveis (100).

Alguns autores mostraram que o vírus estaria presente no sêmen de pacientes mesmo após 3 ou 6 meses do início da infecção e estes mesmos pacientes apresentaram vírus tanto na urina e plasma, quanto na saliva (101–103). Além dos homens, mulheres também parecem ter o sistema genital infectado pelo ZIKV. O vírus foi detectado nas células uterinas através de exame ginecológico, de três a treze dias após as mulheres terem apresentado os sintomas da enfermidade (99,104). Portanto, a duração exata da infecciosidade de fluídos genitais é desconhecida. Estes estudos mostram a gravidade da doença por esta arbovirose, e indicam que é necessária a investigação de uma possível alteração na fertilidade masculina e feminina durante e após a infecção.

Transmissão por transfusão

O ZIKV também pode ser encontrado no sangue dos pacientes infectados (102,105,106) e até mesmo na transfusão de plaquetas já foi relatada sua transmissão (107). Os primeiros relatos de transmissão por transfusão ocorreram na Polinésia Francesa e em Porto Rico em 2014 e 2016, respectivamente (99). Em seguida foram relatados casos no

Brasil e nos Estados Unidos (107,108). Assim como na transmissão sexual, a infecção transmitida por transfusão é de difícil comprovação em áreas endêmicas. Ainda há baixa disponibilidade de ensaios para a detecção do ZIKV e, por isso o número de infecções por transfusão é provavelmente subestimado, com isso, a disseminação do ZIKV se tornou um novo desafio para os bancos de sangue (109). A OMS, a *Food and Drug Administration* (FDA) dos Estados Unidos e a Associação Americana para Bancos de Sangue emitiram recomendações para prevenir infecções transmitidas por transfusão (99). Como a maioria das infecções é assintomática, as estratégias de mitigação mais eficazes para prevenir infecções transmitidas por transfusão são os procedimentos para inativação de patógenos. Um sistema fotoquímico de inativação de patógenos disponível comercialmente tem mostrado inativar uma ampla gama de patógenos, incluindo ZIKV (110).

Transmissão por leite materno

A presença do ZIKV no leite materno já foi estudada e descrita em alguns trabalhos, entretanto nenhum deles confirmou a transmissão (111). Um estudo venezuelano investigou a presença do ZIKV no leite materno em uma paciente que apresentou sintomas como a erupção cutânea maculopapular prurigionosa e conjuntivite, no qual o RNA viral foi detectado por RT-PCR tempo real (112). Em outro trabalho, um caso de infecção por ZIKV foi relatado na Nova Caledônia, relacionada a uma mãe febril que, após 2 dias de parto de um bebê saudável, desenvolveu uma erupção maculopapular. Tanto a mãe quanto o bebê tiveram amostras de sangue coletadas e a mãe também coletou amostras de leite materno. As partículas infectivas do ZIKV foram encontradas apenas nas amostras da mãe (35.000 cópias de RNA/mL no soro e 850.000 cópias de RNA/ mL no leite materno) por meio de RT-PCR (113).

Mesmo no contexto atual do ZIKV, devido ao benefício do leite materno para os bebês, a Organização Mundial de Saúde manteve as recomendações sobre amamentação. Os lactentes devem iniciar a amamentação dentro de 1 hora após o nascimento e continuar por 6 meses exclusivamente e até 2 anos de idade ou mais, após a introdução de alimentos complementares (114). No entanto, mais pesquisas são necessárias sobre a transmissibilidade do ZIKV através do leite materno, e sua persistência.

Transmissão vertical

A transmissão vertical é um dos temas de maior preocupação relacionados ao ZIKV, tendo em vista os casos de microcefalia e outras alterações características da Síndrome Congênita da Zika (SCZ). O primeiro relato da transmissão deste vírus ocorreu durante o primeiro surto na Polinésia Francesa e foi posteriormente confirmada na epidemia do Brasil em 2015 (99,115,116). Apesar de outros flavivírus não terem como característica este tipo de transmissão, sabe-se que a placenta é um órgão alvo para infecções do tipo "TORCH" que incluem toxoplasmose e diferentes vírus, como rubéola, HIV, hepatite B e C, parvovírus, citomegalovírus e outras, que já foram descritas como capazes de destruir a barreira imunológica placentária (117,118). O ZIKV e a infecção congênita sintomática não são observados em todos os fetos expostos, semelhantes ao citomegalovírus e à toxoplasmose (99). A transmissão materno-fetal de ZIKV pode ocorrer em todos os trimestres da gravidez, quer a infecção na mãe seja sintomática ou assintomática (22).

O mecanismo pelo qual o vírus atravessa a placenta para estabelecer a infecção do feto em desenvolvimento ainda não foi completamente elucidado, deste modo, muitos grupos têm tentado esclarecer qual o mecanismo de transposição placentária. Alguns estudos identificaram o genoma do ZIKV no líquido amniótico, no espaço intervilar, decidual e nas vilosidades coriônicas da placenta, além dos tecidos fetais, incluindo o cérebro (24,116,119–121).

Além disso, como relatado na sessão 1.2.3, um estudo *in vitro*, em células isoladas da placenta e linhagens celulares, mostrou que vários tipos celulares placentários (citotrofoblastos, fibroblastos, células deciduais, endoteliais e do sistema imune) possuíam os diferentes receptores de superfície (Tyro3, Axl, Mertk, TIM1, uma imunoglobulina produzida por células T e as proteínas DC-SIGN) aos quais os flavivírus são capazes de se ligar e mediar sua entrada na célula por endocitose (66).

Alguns estudos em culturas de células e tecidos provenientes de mulheres infectadas na gestação têm mostrado que as células da placenta e as células imunes são susceptíveis a infecção por ZIKV (75,122–126).

A prevenção da transmissão materno-fetal é realizada no contexto de evitar ao máximo a exposição da mulher grávida a possíveis picadas de mosquito, com o uso de roupas que cubram a pele e repelentes, assim como a prática sexual segura (99).

Patogênese

Características decorrentes da febre da Zika

A infecção pelo ZIKV exige um diagnóstico diferencial de outras infecções como dengue e chikungunya, pois estas viroses possuem sintomas similares, sendo assim inespecíficos. Os sintomas de Zika geralmente são leves e autolimitados, incluem erupção cutânea maculopapular pruriginosa, febre, poliartralgia, mialgia, vômito, dor de cabeça, prurido, conjuntivite bilateral e edema. Entretanto, a doença é assintomática em aproximadamente 80% dos casos (12,22,127,128). O período de incubação até o aparecimento dos sintomas é de aproximadamente cinco a sete dias. Após o surgimento dos sintomas, estes costumam desaparecer espontaneamente entre três a sete dias (129). Os casos mais graves podem levar a hospitalização e tratamento paliativo afim de aliviar os sintomas e hidratar o paciente com perda de fluido.

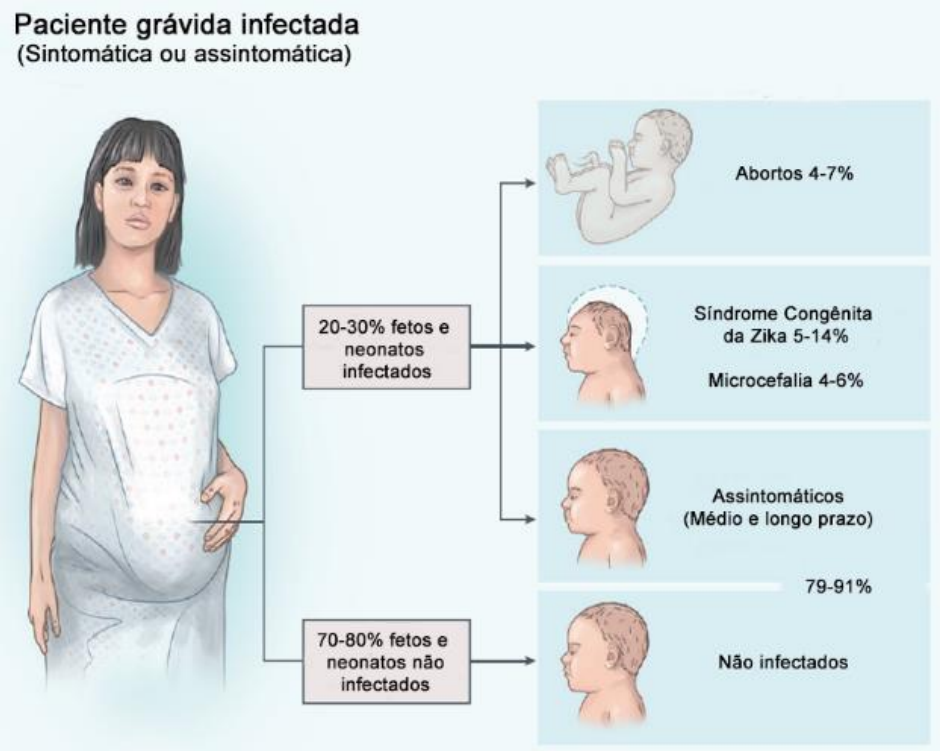
A febre da Zika na maioria das vezes não leva o indivíduo ao óbito, diferente de outras arboviroses como a dengue e chikungunya (27,29). Entretanto, já foi observado que a transmissão vertical do vírus leva a efeitos adversos na gravidez, como anormalidades neurológicas, incluindo a microcefalia nos fetos, lesões articulares e casos de abortos espontâneos e natimortos (4,116,130–132). Adicionalmente, já foi observado que este vírus também pode exercer um efeito patológico neuronal em adultos (22,129,133,134).

Síndrome Congênita da Zika (SCZ) e microcefalia

Estima-se que 20 a 30% das pacientes grávidas infectadas possam transmitir o ZIKV para o feto, dos quais 4 a 7% evoluem a abortos, enquanto de 5 a14% são casos de fetos e neonatos com SCZ (22) (Figura 12). A SCZ consiste em um espectro de falhas congênitas que versam principalmente em manifestações patológicas do sistema nervoso central. Estas incluem na maior parte das vezes: a microcefalia, diminuição do volume cerebral, ventriculomegalia, a ruptura da sequência cerebral fetal, uma condição que decorre de uma interrupção cerebral durante a gestação com subsequente colapso do crânio do feto causado

por diminuição da pressão hidrostática intracraniana, calcificações, sinais piramidais e extrapiramidais, que causam paralisias (incapacidade para realizar movimentos, com perda total da força) ou paresias musculares (dificuldade para realizar movimentos, com perda parcial da força), lesões oculares de atrofia corioretal e pigmentação focal da retina, e contraturas congênitas que parecem ser relacionadas ao processo neurogênico têm sido observadas nos fetos, recém-nascidos e natimortos (22). A maior parte dos estudos relatando as alterações neurológicas são realizadas por exames de imagens, poucos trabalhos mostram a histologia, visto que essas alterações na maioria das vezes não leva o feto à óbito (135–139).

Figura 12 - Esquema com os percentuais de transmissão materno-fetal, e possíveis consequências aos fetos e bebês de mulheres infectadas com ZIKV durante a gravidez



Nota: A transmissão vertical ocorre em 20-30% dos casos de pacientes grávidas infectadas, sejam elas assintomáticas ou não. A SCZ ocorre em 5-14%, enquanto a microcefalia em 4-6%. Dos casos de mães infectadas na gravidez, nascem de 70-91% de bebês assintomáticos ou não infectados.
Fonte: Adaptada de Musso et. al. (22).

De acordo com os boletins epidemiológicos realizado pelo Sistema Único de Saúde, no Brasil, foram notificados 18.687 casos suspeitos de Síndrome Congênita da Zika, dos quais 3.512 (18,8%) foram confirmados por exames, desde 2015 até a semana 5 de 2020 (29,34,130). Considerando apenas o ano epidemiológico de 2020, 93 novos casos foram notificados, dos quais sete (7,5%) foram confirmados, sendo: dois nascidos em 2018, quatro

em 2019 e um em 2020. Portanto, muito embora o período de emergência tenha sido encerrado, novos casos de SCZ continuam ocorrendo no país.

Na epidemia de ZIKV no Brasil em 2015, houve um grande número de casos de microcefalia, rapidamente associados à infecção, visto que estes casos correspondiam às áreas de surto da doença (131,140–142). A microcefalia congênita é uma condição caracterizada pela redução da circunferência da cabeça, associada ao desenvolvimento do cérebro que pode ter sido retardado ou interrompido, gerando algum tipo de deficiência intelectual (4,143). Em 2017, o Ministério da Saúde adotou novos parâmetros para medir o perímetro cefálico e identificar casos suspeitos de microcefalia, seguindo a recomendação da OMS. Para menino, a medida é igual ou inferior a 32,5 centímetros e, para menina, igual ou inferior a 31,5 centímetros. Para bebês nascidos com menos de 37 semanas de gestação (prematuros) é utilizada a tabela de *InterGrowth*, que tem como referência a idade gestacional do bebê (130).

Estudos em modelos animais mostram que o ZIKV possui tropismo cerebral, causando doença neuronal, microcefalia pós-natal e vários déficits comportamentais na vida adulta (2,144). Na fase aguda da infecção, os camundongos desenvolveram convulsões frequentes, que foram reduzidas pela inibição de TNF-α. A replicação do ZIKV persistiu em camundongos adultos, neonatais infectados, e os animais mostraram maior suscetibilidade a convulsões quimicamente induzidas, neurodegeneração e nos resultados histopatológicos, áreas com infiltrado inflamatório, necrose, microgliose, desorganização celular e calcificações cerebrais (144). Já em macacas *Rhesus* infectadas no início da gravidez, houve viremia materna prolongada, incluindo perda fetal, e neuropatologia fetal, menor tamanho cerebral e lesões histopatológicas, incluindo microcalcificações, hemorragia, necrose, vasculite, gliose e apoptose de células neuroprogenitoras (145).

Estudos recentes em culturas de células mostram que células neurais possuem o receptor Axl, que seria um bom receptor para a entrada do ZIKV nas células, o que as torna suscetíveis à entrada do vírus (146). Além disso, o ZIKV pode infectar diretamente as células-tronco neuronais do feto e prejudicar o crescimento do cérebro. A ativação da proteína P53 e a inibição da via mTOR pela infecção por ZIKV em células-tronco neuronais induzem alterações bioquímicas nas células, como a mudança da glicólise para a fosforilação oxidativa, levando a diferenciação imatura, apoptose e exaustão de células-tronco (63). Os efeitos da infecção por ZIKV em células-tronco neurais humanas crescendo como neuroesferas e organóides cerebrais também foram estudados. O ZIKV induziu morte celular nos organóides, alterando sua morfologia e inibindo a neurogênese em um curto período de tempo, entre 3 e 6 dias (147).

Nos estudos em humanos, vinte e quatro crianças nascidas com SCZ no surto de 2015 foram acompanhadas para a avaliação do seu neurodesenvolvimento no segundo ano de vida. As crianças apresentaram circunferência cefálica abaixo para idade (levando em consideração também o sexo), na qual a taxa de crescimento até o segundo ano de vida foi de 10,3 cm (crescimento esperado de 13 cm). O tônus muscular foi aumentado em 23 (95,5%) das 24 crianças, os reflexos musculotendinosos aumentaram em toda a amostra e o clônus (movimento muscular involuntário) esteve presente em 18 (77,3%) das 24 crianças. Todas as crianças, exceto uma, tiveram epilepsia. As crianças apresentaram também comprometimento do desenvolvimento neuropsicomotor; nenhuma delas conseguiu ficar de pé com o apoio, caminhar ou falar (148). Outro estudo com 34 crianças com SCZ e também no segundo ano de vida, mostrou que os indivíduos apresentavam alterações anatômicas como calcificações puntiformes, redução difusa do volume encefálico, hipodensidade da substância branca dos hemisférios cerebrais e acentuação dos sulcos entre as dobras cerebelares. As funções neuromotoras também estavam comprometidas, 94,1% dos participantes não conseguiram realizar movimentos motores finos adequados à idade e 70,6% não conseguiam andar (149). Em ambos estudos, foi observada uma grande capacidade de socialização e reconhecimento da família, sugerindo que além dos cuidados de saúde com equipes multiprofissionais, a participação familiar e o ambiente doméstico é essencial para a melhor qualidade de vida das crianças com SCZ (148,149).

Abortos espontâneos e natimortos

Os abortos espontâneos podem ocorrer em consequência da infecção materna, sintomática ou mesmo assintomática. Alguns trabalhos detectaram a presença do vírus nas placentas e tecidos fetais coletados de abortos, por PCR tempo real ou hibridização *in situ*, ainda em fase inicial da gestação, com 8, 10 ou 11 semanas (150–152).

Um estudo realizado após o surto no Brasil, mostra a análise histopatológica de um natimorto, coletado após um aborto retido com idade gestacional de 8 semanas, que apresentou áreas de lesão necrótica na porção fetal próximo ao saco vitelino. Outros bebês nascidos mortos, ou que vieram a óbito em até 48h após o parto tiveram muitas alterações em diferentes órgãos avaliados, na maior parte das vezes, essas mudanças não são encontradas em mais de um caso, sendo denominadas não patognomônicas. Ou seja, as modificações ocorrem

de forma não determinante, sem sinais próprios específicos que possam ser característicos à infecção por ZIKV (152).

Os cérebros dos bebês exibiram edema, calcificação, infiltrado inflamatório, necrose ou apoptose, entre outras modificações. Nos fígados foram encontradas áreas de autólise, esteatose e infiltrado inflamatório. Os rins possuíam congestão vascular difusa ou na porção medular e imaturidade dos glomérulos, mesmos nos nascidos à termo. Nos pulmões, foram observadas áreas de edema, congestão e hemorragia e outras alterações. Foram ainda, investigados outros órgãos como baço e coração, todos apresentando algum tipo de dano. Nos diferentes casos, foram encontrados antígenos de ZIKV em diferentes órgãos por imunohistoquímica ou RT-PCR tempo real (152).

Síndrome de Guillain- Barré causada por ZIKV

A primeira ocorrência grave de infecção pelo ZIKV em adultos foi a Síndrome de Guillain-Barré (SGB). A síndrome de Guillain-Barré é uma doença auto-imune na qual o sistema imunológico ataca parte do sistema nervoso periférico, causando formigamento, fraqueza muscular, paralisia e até a morte (153). A ligação entre o ZIKV e a SGB foi confirmada por um estudo de caso realizado na Polinésia Francesa, quando 42 pacientes desenvolveram a síndrome durante o surto de Zika (154). A maior parte dos pacientes (88%) apresentou o quadro característico de SGB rapidamente, após 6 dias de infecção. A incidência da SGB durante os surtos de ZIKV varia de país para país, e foi 20 vezes a linha de base na Polinésia Francesa e dez vezes a linha de base na Venezuela (155,156). Na maior série de SGB associada ao ZIKV que ocorreu na Polinésia Francesa e na Colômbia, as principais características da síndrome foram uma progressão rápida para o nadir (cerca de 1 semana), uma fase de platô curto (de aproximadamente 4 dias), e uma alta proporção de paralisia facial. Os achados eletrofisiológicos mostraram que a maioria dos casos polinésios franceses era do subtipo de neuropatia axonal motora aguda (AMAN), enquanto na Colômbia o subtipo predominante era a polirradiculoneuropatia desmielinizante inflamatória aguda (AIDP), que é o subtipo mais comum de Guillain-Barré (154,157). Em março de 2017, 23 países ou territórios localizados principalmente na América do Sul, relataram um aumento na incidência da SGB ou confirmação laboratorial de uma infecção pelo ZIKV entre os casos de síndrome de Guillain-Barré (158).

A patogênese da SGB associada ao ZIKV ainda é desconhecida: mecanismos neuropatogênicos diretos, resposta imune hiperaguda, desregulação imune e mimetismo molecular contra antígenos nervosos são ainda hipóteses a serem mais estudadas (159). O que se sabe até o momento é que o quadro patológico clássico da síndrome de Guillain-Barré na AIDP é a presença de células mononucleares multifocais em todo o sistema nervoso periférico, em que a distribuição desta inflamação leva ao déficit clínico. De acordo com uma hipótese, os macrófagos ativados são direcionados para antígenos na superfície das células de Schwann ou na bainha de mielina por linfócitos T ativados, assim desnudando os axônios. De acordo com uma hipótese alternativa, mas não mutuamente exclusiva, o evento inicial seria a ligação de anticorpos à superfície da célula de Schwann, com a fixação do complemento, levando ao provável dano na célula de Schwann e dissolução da mielina. Evidências para essa teoria vêm de material de autópsia realizados no início do curso da doença. Em lesões mais graves, os axônios também são danificados, provavelmente como uma consequência secundária das enzimas tóxicas e radicais livres liberados pela resposta inflamatória imunomediada dirigida contra a mielina (160).

Já na patogênese da AMAN, o processo é um pouco diferente. Sendo o alvo na ligação mediada por receptor Fc de anticorpos direcionados contra antígenos de gangliosídeos no axolema, os macrófagos invadem os nódulos de Ranvier (regiões limites entre uma célula de Schwann e outra) onde se inserem entre o axônio e o axolema de células de Schwann circundante, deixando a bainha de mielina intacta. Em casos graves, os axônios são danificados na raiz ventral, o que pode causar degeneração grave de todo o axônio. Entretanto, os pacientes com AMAN geralmente atingem seu nadir mais cedo e se recuperam tão rapidamente quanto aqueles com AIDP. Esse rápido declínio e subsequente recuperação pode ser porque na AMAN o processo patológico bloqueia a condução, mas não rompe o axônio (160).

Ainda não se sabe ao certo se na infecção por ZIKV o quadro de SGB é causado por estas repostas inflamatórias nas quais as células T e mononucleares são ativadas gerando um quadro auto-imune, ou se o vírus infectaria diretamente o sistema nervoso periférico, visto que possui tropismo por células do sistema nervoso (161).

O tratamento da SGB requer instalações de tratamento intensivo, transfusão de plasma e administração de imunoglobulinas intravenosas, sendo uma terapia desafiadora em países com poucos recursos (159).

Patogênese da placenta nos casos de ZIKV

Apesar dos esforços recentes em elucidar as consequências da infecção por ZIKV no contexto materno-fetal e da transmissão vertical, poucos trabalhos investigando a placenta infectada foram publicados e ainda há muito a ser estudado. Nos primeiros trabalhos após os grandes surtos de 2015, autores elaboraram duas hipóteses sobre o papel da placenta. A primeira é que a placenta poderia transmitir diretamente o ZIKV ao embrião ou feto. Alternativamente, a própria placenta poderia estar montando uma resposta à exposição e esta resposta poderia contribuir para a deformidade neurológica do feto (162).

Com o conhecimento gerado após estes surtos, hoje sabemos que o ZIKV é capaz de infectar células da placenta, que seriam então transmitidas para o feto (66). Entretanto, como relatado na sessão 1.4.5, o modo como ocorre essa transmissão permanece em discussão, assim como as consequências desta infecção na própria placenta e a sua implicação no desenvolvimento do feto. Há relatos sobre a identificação do genoma do ZIKV no líquido amniótico, no espaço intervilar, decidual e nas vilosidades coriônicas da placenta (24,116,119–121).

A histopatologia da placenta infectada por ZIKV foi pouco descrita até o momento e o estudo dos tecidos infectados poderia auxiliar o entendimento de como ocorre a transmissão vertical e os efeitos da infecção na própria placenta ou até mesmo ao feto. Um estudo realizado em placenta prematura demonstrou a proliferação das células de Hofbauer, aumento nas vilosidades coriônicas além de edema e hipercelularidade no estroma. A presença do ZIKV foi detectada nas células do estroma e das vilosidades coriônicas por hibridização *in situ*, mostrando que as células placentárias foram não apenas permissivas à infecção, mas também à replicação viral. Este é um dos resultados que levam a crer que o ZIKV é capaz de ter acesso ao feto após a infecção e proliferação nas células placentárias. Entretanto, neste estudo não foram detectadas áreas de necrose, fibrose e outros tipos de alterações (123).

A análise de placentas infectadas nos três diferentes trimestres mostrou edema do estroma nas vilosidades coriônicas, com dilatação irregular e agregados proeminentes de células vacuoladas. As membranas fetais também mostraram várias alterações reativas, incluindo um aumento no número de células mononucleares vacuoladas no tecido conjuntivo subamniótico, pelo aumento de macrófagos (126). Beaufrère et al. também descreveram alterações características de inflamação na análise histopatológica de três placentas de pacientes diagnosticadas com Zika. As placentas possuíam corioamnionite, vilosite e

intervilite (inflamação nas membranas, vilosidades coriônicas e intervilo, respectivamente), com infiltrado inflamatório, hiperplasia de células de Hofbauer e espessamento de endotélio (125). A análise histológica das culturas explantadas infectadas *ex vivo* com ZIKV mostrou alterações drásticas na arquitetura das vilosidades placentárias com ruptura das camadas trofoblásticas e alterações na morfologia dos núcleos, como cariorrexe e cariólise (75).

Apesar de descrevem uma inflamação placentária, raros trabalhos abordam com detalhes o perfil imunológico da placenta no contexto da infecção por Zika. Um estudo recente descreve a hiperplasia das células de Hofbauer e numerosas células histiocíticas na porção materna, além de deciduíte com predominância de linfócitos. Neste caso, houve um aumento de linfócitos T CD3+, bem como de células T CD4+ e T CD8+ (163). Ainda corroborando a ideia de que o ZIKV chegaria até o feto pela infecção célula a célula, um estudo *in vitro* com células isoladas de placenta madura mostrou que os macrófagos placentários (células de Hofbaeur) e citotrofoblastos são permissivos a infecção pelo ZIKV, sendo os citotrofoblastos um pouco mais resistentes à infecção. As células de Hofbauer são estimuladas a apresentar uma resposta pós-infecção, com aumento da secreção de IFN-α e das citocinas pró-inflamatórias IL-6, IL-10 e MCP-1. Tanto macrófagos como os citotrofoblastos infectados apresentaram um aumento nos níveis de transcritos antivirais após a infecção por ZIKV (164).

Perfis imunológicos distintos foram detectados em diferentes trimestres no soro de mulheres grávidas infectadas por ZIKV, mas as quimiocinas CXCL10, CCL2 e CCL8 foram especificamente associadas à infecção sintomática por ZIKV durante a gravidez (165).

Devido à grande atenção decorrente das alterações nos fetos, alguns trabalhos têm sido feitos para estabelecer um modelo animal de infecção com ZIKV a fim de facilitar o entendimento da transmissão vertical e viabilizar testes terapêuticos e vacinais (121,166–169). Em uma dessas investigações, Miner et al. (121) inocularam ZIKV por via subcutânea em animais permissivos à infecção (camundongos heterozigotos *Ifnar* $^{+/-}$, gene que codifica o receptor do IFN1). Muitos fetos dos animais estudados exibiram restrição de crescimento intrauterino e vieram à termo com tamanho 20% menor, além de vários fetos terem sofrido morte placentária e reabsorção. Adicionalmente, foram identificadas também outras alterações decorrentes da infecção, como palidez e áreas de tecido necrótico na placenta (121). Um trabalho semelhante, usando camundongos heterozigotos *Ifnar* $^{+/-}$ e homozigotos *Ifnar* $^{-/-}$ mostrou que o título viral e a replicação na placenta é maior nos animais homozigotos, que não expressam o receptor para IFN. Estes animais também tiveram alterações no

desenvolvimento e morfologia da placenta (166). Esses resultados mostram que o IFN tipo I é importante como um possível mediador de complicações na gravidez.

Com base nessas informações, pode-se perceber a importância de uma melhor investigação das consequências que o ZIKV causa nas placentas das mulheres infectadas na gestação. O estudo histopatológico pode melhorar compreensão dos danos ao próprio tecido placentário, essencial na gestação, e das suas consequências nos danos fetais.

Estresse oxidativo causado pela infecção por ZIKV

As infecções pelos flavivírus desencadeiam o estresse oxidativo, que afeta tanto o metabolismo celular como o ciclo de vida do próprio vírus. O estresse oxidativo ocorre quando o equilíbrio homeostático de geração e neutralização das espécies reativas de oxigênio (ROS, do inglês *reactive oxygen species*) é perdido na célula, levando ao estado anômalo e induzindo alterações prejudiciais ao microambiente celular (170).

As mitocôndrias são responsáveis pela a maior parte do consumo de oxigênio dentro da célula, para a produção da energia celular, entretanto, são também as principais produtoras de ROS na célula. A respiração mitocondrial depende de transferência de elétrons e de um gradiente de prótons para conduzir a síntese de ATP, e neste processo, as ROS são produzidas como subprodutos naturais. Uma das espécies mais produzidas é o superóxido, formado por um eletrón em fuga das cadeias de transporte de elétrons nas mitocôndrias e pela oxidação de nicotinamida-adenina dinucleotídeo fosfato (NADPH) por NAPH oxidases. O superóxido produzido é rapidamente convertido em peróxido de hidrogênio (H_2O_2) e em radicais hidroxila, por enzimas superóxido dismutases (SODs) que estão na mitocôndria e no citoplasma (170). Em um segundo nível de defesa, a catalase (CAT), enzima localizada no peroxissomo, desempenha um papel crucial na resposta adaptativa ao H_2O_2, pois, apesar de menos nocivo, na presença de Fe^{2+}, algumas dessas moléculas podem ser reduzido a OH^-, altamente reativos que atacam várias biomoléculas. A catalase decompõe o H_2O_2 em oxigênio e água, que são inofensivos à célula (171).

O óxido nítrico (ON), uma espécie reativa de nitrogênio, é produzido através da conversão de L-arginina em ON através de ON sintases (NOS, do inglês *NO synthases*), existindo em estado gasoso. O aumento de ON é tóxico as células, visto que o produto da

reação de ON com os ânions superóxido é o peroxinitrito, que é altamente reativo e pode gerar danos graves dentro e fora da célula, já que são lipossolúveis (172).

A peroxidação de ácidos graxos leva a produção de malondialdeído (MDA), o principal e mais estudado produto de peroxidação de lipídeos. A produção de MDA ocorre nas condições de estresse, sendo capaz de interagir com as bases nitrogenadas de ácidos nucleicos, podendo levar a mutações e ligações proteína-DNA (173).

Muitas infecções virais levam ao estresse oxidativo, prejudicando a fisiologia da célula hospedeira, dentre elas, está a infecção pelo ZIKV, entretanto, os trabalhos investigando o estresse oxidativo decorrente dessa infecção são escassos. O que se sabe atualmente é que o estresse oxidativo causado por ZIKV leva a alterações na tradução do RNA produzido pela célula, consequentemente à montagem de grandes agregados compostos pelos complexos de pré-iniciação de tradução parados, denominados grânulos de estresse, além de desencadear o processo de morte celular (174,175).

Uso de linhagens de células para os estudos *in vitro*

Uso de linhagem de células de mastócitos

Os mastócitos são células imunes abundantes na placenta e que possuem papel importante nas reações imunológicas em geral (94,95). Por estar presente na pele e localizados sempre próximos aos vasos sanguíneos, os mastócitos podem estar entre as primeiras células imunes infectadas pelo ZIKV após o repasto do mosquito na pele. Os sintomas mais frequentes da Zika incluem erupção cutânea e prurido, que são aliviados pela administração de medicamentos antialérgicos (anti-histamínicos), o que nos leva a acreditar que os mastócitos podem desempenhar um papel, ainda não elucidado, na patogénese da doença (176). Pela localização na placenta, poderia ser uma das células envolvidas na transmissão vertical na infecção placentária, entretanto ainda não existem trabalhos que explorem sua suceptibilidade e permissividade à esse vírus.

Embora não haja trabalhos na literatura investigando o envolvimento de mastócitos na infecção por ZIKV até o momento, sua atuação em outras arboviroses, como a dengue, já foi provado algumas vezes. Seus mediadores secretados são encontrados em níveis elevados,

especialmente em pacientes com extravazamento de plasma (177,178). Os mastócitos são permissivos à infecção pelo vírus da dengue, mas é provável que haja menos receptores específicos, já que a quantidade de vírus para infectar este tipo celular é sempre maior em comparação com a quantidade usada para macrófagos e células dendríticas (177,179,180).

As células HMC-1 são uma linhagem de mastócitos humanos, que liberam diferentes mediadores e citocinas após estímulos de ativação e degranulação. Esta linhagem possui as características necessárias de um modelo *in vitro* para o desenvolvimento de estudos em mastócitos (181). As células HMC-1 são amplamente utilizadas em estudos de degranulação, envolvimento na ativação endotelial e interação com outros arbovírus (182–184).

Uso de linhagens de células placentárias

Algumas linhagens celulares foram desenvolvidas a fim de estudar o desenvolvimento e fisiologia da placenta, incluindo as células BeWo, células JEG-3, JAR e HTR-8/SVneo (185). A linhagem celular HTR-8/SVneo foi desenvolvida a partir de células trofoblásticas extravilosas humanas que foram transfectadas com o antígeno T do vírus símio 40 (SV40). A transfecção confere à célula uma capacidade de divisão ilimitada, ao mesmo tempo em que mantém as características de células trofoblásticas extravilosas humana (186). Por essa razão a linhagem celular HTR-8/SVneo é considerada um modelo apropriado para a realização de estudos *in vitro* sobre a interface materno-fetal.

As células HTR-8/Svneo já foram testadas em dois estudos sobre suceptibilidade e permissividade pelo ZIKV, sendo estas aptas tanto a entrada quanto a replicação viral (122,124).

Sendo assim, pode-se perceber a importância de uma melhor investigação das consequências que o ZIKV causa nas células placentares. O estudo *in vitro* é necessário e uma excelente ferramenta para melhorar compreensão de susceptibilidade e permissividade do ZIKV, e dos danos que o vírus pode causar nas células infectadas.

Diagnóstico, vacinas e tratamentos

O diagnóstico de Zika hoje é realizado, inicialmente, através de anamnese e identificação dos sintomas do paciente, entretanto, a febre do Zika pode facilmente ser confundida com outras doenças circulantes nas mesmas regiões como dengue e chikungunya. Por este motivo, diferentes testes de diagnóstico estão disponíveis para ajudar a determinar se uma pessoa está infectada com a doença causada pelo ZIKV. Os testes sorológicos são encontrados em diversos laboratórios e a sorologia para IgM e os anticorpos neutralizantes específicos de ZIKV geralmente podem ser detectados próximo ao fim da primeira semana da doença. Os níveis de IgM são variáveis, mas geralmente são positivos a partir do quarto dia após o início dos sintomas e continuam por até 12 semanas após o início dos sintomas ou exposição, podendo persistir por mais tempo. Nas gestantes sintomáticas, a sorologia IgM é realizada simultaneamente com o NAT (teste de ácido nucleico), no qual é feito o RT-PCR tempo real para RNA de dengue, Zika e chikungunya, realizado em amostras de soro e urina pareadas. Entretanto, após o surto de Zika, foi observado que os testes com amostras de pacientes fora do período de viremia são na maioria dos casos, negativo. Ou seja, um resultado de teste NAT negativo não elimina a hipótese de infecção pelo ZIKV (22,187,188).

Outro teste, como o Ensaio Imunossorvente por Ligação Enzimática de Captura de Anticorpos IgM para Zika (MAC-ELISA) pode ser usado para a detecção de anticorpos IgM para o vírus no soro, no entanto, devido à reação cruzada com outros flavivírus e possível reatividade não específica, os resultados podem ser difíceis de interpretar (188).

Atualmente, não existem terapias ou vacinas disponíveis contra o ZIKV. Infelizmente, o ZIKV apresenta diversos fatores que são considerados grandes desafios para o desenvolvimento de fármacos e vacinas. Um desses fatores é a necessidade de direcionar os fármacos e as vacinas para populações imunocomprometidas, como as mães grávidas e seus fetos, além do risco de estimular respostas imunitárias prejudiciais (aumento da resposta autoimune ou dependente de anticorpos da infecção naqueles com exposição anterior a flavivírus). Outra preocupação é em relação aos casos assintomáticos, em que a infecção silenciosa pode atrasar o tratamento e aumentar o risco de transmissão para outros por múltiplas vias de transmissão (através do vetores artrópodes, via sexual, sanguínea e potencialmente por outros fluidos corporais).

O tratamento atualmente consiste apenas no alívio dos sintomas usando analgésicos e anti-inflamatórios para reduzir a dor nas articulações e nos músculos, antialérgicos para alívio

do prurido e exantemas e colírios três a seis vezes ao dia como lubrificante. É importante que o paciente faça repouso por sete dias, coma alimentos ricos em minerais e vitaminas, e beba bastante líquido para uma recuperação rápida (189).

Muitos grupos têm trabalhado arduamente no desenvolvimento de diferentes modelos vacinais contra o ZIKV. Atualmente, existe mais de 30 modelos vacinais sendo desenvolvidos em todo o mundo (190). Os testes pré-clínicos mostram resultados eficazes de vacinas inativadas, atenuadas e de DNA (190–192).

Os avanços nas vacinas para a prevenção desta doença têm tido um ritmo inédito e após um ano do começo do surto de Zika no Brasil, já existia uma vacina de DNA com resultados pré-clínicos promissores e que atualmente está na fase I de testes clínicos. Essa vacina foi desenvolvida com genes que codificam as proteínas virais prM e E, estas proteínas são antígenos flavivirais escolhidos com frequência como alvos vacinais, já que fazem parte da estrutura do vírus, o que geraria uma resposta imune rápida. Essa resposta seria baseada em anticorpos neutralizantes, capaz não só de proteger contra a infecção, mas também de neutralizar e diminuir a viremia na fase aguda da infecção. Nos testes em macacos *Rhesus,* 17 dos 18 macacos foram protegidos após a vacinação e o desafio (193).

Os doentes com ZIKV sem complicações geralmente não são encaminhados para terapia, uma vez que os sintomas normalmente desaparecem dentro de 3-7 dias. O único tratamento disponível para a infecção é paliativo. Febre e/ou artralgia podem ser tratados com paracetamol e prurido com drogas anti-histamínicas. Recomenda-se também uma adequada reidratação para a perda de fluido. Aspirina e anti-inflamatório não-esteroides não são recomendados devido à possibilidade de diagnosticar erroneamente infecções como DENV (129).

Algumas estratégias com fármacos antivirais têm sido testadas. Foi observado que a cloroquina, um composto já utilizado no tratamento da malária, é capaz de reduzir o número de células infectadas com ZIKV *in vitro* e inibe a replicação do vírus e a morte celular promovida pela infecção sem efeitos citotóxicos. Entre os diferentes tipos celulares testados estão a linhagem de células Vero, células endoteliais microvasculares cerebrais e também células tronco neurais. A cloroquina apresenta resultados bastante interessantes, pois atuaria nos estágios iniciais, impedindo a infecção desde o começo, no momento da endocitose (194).

Nestes estudos visando tratamentos, há uma grande preocupação em relação a infecção neonatal, por causa das complicações neuropatológicas. Estudos em camundongos sugerem que a inibição precoce da neuroinflamação mediada por TNF-α, pode ser uma estratégia

terapêutica eficaz para prevenir o desenvolvimento de anormalidades neurológicas crônicas, ainda na gestação (144).

Um componente inibidor da polimerase já comprovadamente eficaz contra a hepatite C, o sofosbuvir, que também foi testado *in vitro* em células de linhagem de placenta (Jar) e hepáticas (Huh-7) e *in vivo* em camundongos, parece inibir a infecção por ZIKV (195). A busca por novos tratamentos levou um grupo a desenvolver uma polimerase expressa e purificada em *E. coli* que seria capaz de inibir a infecção de ZIKV e outros vírus (196). Ou seja, estes trabalhos mostram que as estratégias atuais usadas para desenvolver tratamentos de ZIKV seguem os métodos comprovados já usados anteriormente contra outros patógenos.

Entretanto, uma vez que nenhum agente antiviral tenha sido aprovado por agências reguladoras para o tratamento da infecção pelo ZIKV, o tratamento clínico da infecção aguda pelo ZIKV segue sendo feito apenas com cuidados de apoio (22).

Por tudo que foi relatado até o momento, pode-se notar que ainda são escassos os trabalhos sobre a placenta no contexto da infecção por ZIKV, sendo necessário maior aprofundamento nos possíveis danos causados neste órgão, além do contexto em relação a resposta imune e inflamação frente à infecção. Desde 2015 enfrentamos este agravo no Brasil, com preocupantes consequências para os fetos na gestação, que podem desenvolver a SCZ, ou até mesmo casos de abortos, desfechos dolorosos e complicados para os indivíduos envolvidos.

Diante deste cenário, o presente trabalho foi conceitualizado a partir do panorama vivenciado no nosso país e no nosso estado, com o surto de Zika em 2015-2017, que levou à circunstância de atenção com as pacientes grávidas, pelo grande risco à gravidez e ao feto. Neste, visamos analisar os dados clínicos e laboratoriais de gestantes que foram infectadas nos diferentes trimestres, assim como a replicação de ZIKV nas placentas, seu perfil histopatológico e imune. Além disso, analisamos a histopatologia e replicação viral em diferentes órgãos de um natimorto. Por fim, utilizamos os experimentos *in vitro* para elucidar questões como o envolvimento de uma célula imune específica, o mastócito, na infecção por ZIKV e as possíveis consequências da infecção nas próprias células placentárias humanas.

1 **OBJETIVO**

1.1 **Objetivo geral**

Investigar a imunopatogênese e os aspectos ultraestruturais das placentas de pacientes infectadas por ZIKV na gestação, a histopatologia e replicação viral em órgãos de um natimorto; além de avaliar a susceptibilidade, permissividade e consequências do ZIKV em linhagens de mastócitos e células trofoblásticas *in vitro*.

1.2 **Objetivos específicos**

Em relação às gestantes e ao natimorto:

a) investigar se houve replicação viral nas células das placentas das gestantes infectadas por ZIKV e nos órgãos de um natimorto pela detecção da proteína NS1; além de detectar outros antígenos virais pelas técnicas de imunohistoquímica, imunofluorescência e/ou hibridização *in situ*;
b) avaliar os aspectos clínicos materno-fetais, por meio de exames de imagem e bioquímicos laboratoriais, além de RT-qPCR e PRNT nos soros;
c) analisar as alterações histopatológicas (placenta e órgãos do natimorto) e na matriz extracelular (placenta), pelas colorações de Hematoxilina-Eosina, Picro Sirius Red e/ou Ácido Periódico Schiff observados em microscopia óptica;
d) analisar a ultraestrutura das células placentárias, observadas em microscópio eletrônico de transmissão;
e) avaliar a resposta imunológica pela detecção de células do sistema imune, citocinas e mediadores, por imunohistoquímica e imunofluorescência.

Em relação às linhagens de células infectadas *in vitro*:

a) avaliar a susceptibilidade e permissividade de mastócitos e células trofoblásticas ao ZIKV, por imunofluorescência e/ou citometria de fluxo;
b) analisar os mastócitos infectados, nos seguintes parâmetros: degranulação, produção de citocinas e mediadores, por ensaio colorimétrico e ELISA;
c) analisar os aspectos ultraestruturais de mastócitos e células trofoblásticas infectadas, observados em microscópio eletrônico de transmissão;
d) avaliar o estresse oxidativo nas células trofoblásticas infectadas a partir da atividade de enzimas e quantificação das espécies reativas, por ensaios colorimétricos.

2 RESULTADOS

Os resultados desta tese estão divididos em cinco partes, escritas na forma de artigos científicos, que já foram publicados, submetidos ou que estão sendo finalizados para submissão. Eles serão listados abaixo na ordem em que serão discutidos.

2.1 Placental histopathology and clinical presentation of severe Congenital Zika Syndrome in a Human Immunodeficiency Virus-exposed uninfected infant (Artigo publicado)

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Placental Histopathology and Clinical presentation of severe Congenital Zika syndrome in a Human Immunodeficiency Virus-exposed Uninfected Infant

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In the large Zika virus (ZIKV) epidemic that occurred in Brazil in 2015, the intrauterine fetal exposure to ZIKV was associated with a significant risk of developing microcephaly and neurological disorders in the infected infants. ZIKV-associated disease has since been reported in 24 countries in the Americas. At present, definitive evidence is lacking regarding the intrauterine co-exposure to ZIKV and other viral infections and whether the coinfection impacts the risk of acquiring either infection or disease severity. Here, we provide evidence of intrauterine exposure to both ZIKV and human immunode-ficiency virus (HIV) infections, causing congenital Zika syndrome in an HIV-exposed uninfected infant. Clinical, imaging and laboratory examinations of the pregnant woman and the newborn were performed. Histopathology, ZIKV/HIV-specific immunoassays, and ultrastructural evaluation of the placenta were performed. The Zika-asymptomatic, HIV-positive pregnant woman underwent ultrasounds revealing fetal cerebral ventricu-lomegaly, microcephaly, and brain atrophy. Her baby girl was born small for gestational age and with the neurological sequelae of congenital Zika syndrome. The evaluation of the abnormally large term placenta revealed severe damage to the maternal decidua and chorionic villi, cells positive for ZIKV-specific antigens but not for HIV antigens, and intracellular membranous clusters of virus-like particles approximately 25 nm in diameter. The rapid progression and severity of the congenital Zika syndrome may be related to the uncontrolled HIV disease in the mother. The poor inflammatory response observed in the placenta may have reduced the inherent risk of mother-to-child trans-mission of HIV.

Keywords: Zika virus, placenta, congenital Zika syndrome, histopathology, microcephaly, human immunodeficiency virus

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INTRODUCTION

The enveloped, positive-strand RNA Zika flavivirus (ZIKV) can be transmitted to humans through *Aedes* mosquito bites, from mother to child, by unprotected sexual intercourse and by blood transfusion (1). The transplacental transmission of ZIKV is highly neurotropic and teratogenic, resulting in severe congenital microcephaly and a broad spectrum of gross and microscopic neuropathologic abnormalities (2, 3). The estimated absolute risk of a notified microcephaly case varies from 0.03 to 17.1% depending on geographical area, the definition of microcephaly used and the ZIKV infection rate (4).

The ZIKV particle is approximately 25–30 nm and shares many structural similarities with other flaviviruses such as Dengue, West Nile, Japanese encephalitis, and Yellow Fever (5). Its viral genome encodes a polyprotein precursor that is processed into the structural proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (6). The human immuno-deficiency virus (HIV), which belongs to the *Retroviridae* family, features two distinct genotypes, HIV-1 and HIV-2. HIV particles are approximately 120 nm in size and contain two single-stranded RNA copies of 9.2 kb (7).

Within less than 2 years from the emergence of an epidemic of ZIKV in Brazil in 2015, 24 countries in the Americas and the Caribbean have reported cases of microcephaly associated with the mother-to-child intrauterine transmission of ZIKV (8). The cellular and molecular pathways that ZIKV uses to breach the human placental barrier to reach the embryo have not been fully elucidated. ZIKV RNA has been detected in various samples from infected individuals: the amniotic fluid, intervillar space, decidua, and chorionic villi (CV) of the placenta, in addition to fetal tissues (9–12). Other placental cells [syncytiotrophoblasts (STB), cytotrophoblasts (CTB), decidual and endothelial cells, macrophages, and dendritic cells] are also permissive to ZIKV (13, 14). At present, there is no decisive proof of intrauterine co-exposure to ZIKV and other viral or parasitic infections and no data on whether such co-exposure impacts the risk of infection or disease severity. One recent report described a case of ZIKV infection acquired during the first trimester in an HIV-infected pregnant woman from Brazil, which was associated with con-genital defects and fetal demise. Unfortunately, evaluation of the placenta was not performed (15).

Here, we provide evidence of the intrauterine exposure to both ZIKV and HIV, infection of the placenta with ZIKV, and the outcome of severe congenital Zika syndrome in an HIV-exposed uninfected infant.

CASE AND METHODS

Clinical Presentation

A 32-year-old, HIV-positive, primiparous woman from the Northwestern region of the State of Rio de Janeiro, Brazil, had an ultrasound exam at 17 weeks of gestation that was unremarkable about her female fetus. Three weeks later, a second ultrasound revealed fetal cerebral ventriculomegaly. At 24 weeks of gestation, a third ultrasound demonstrated microcephaly and brain atrophy with non-visualization of the cavum septi pellucidi. Prenatal serology was negative for IgM against TORCH antigens (Table S1 in Supplementary Material). The ZIKV infection in the pregnant woman was asymptomatic, but she cited having been bitten by mosquitoes during pregnancy. Two weeks before delivery, the mother had an HIV viral load of 9,323 copies/mL and a CD4+ T-lymphocyte count of 373 cells/μL. Since her HIV diagnosis in the year 2012, the woman had occasionally used antiretroviral treatment consisting of Tenofovir, Lamivudine, and Lopinavir/ Ritonavir, but she did not have any AIDS-defining illness. Before delivery, she received Zidovudine for the prevention of mother-to-child transmission of HIV. At 38 weeks of gestation (June 1st, 2016), her baby girl was born by cesarean delivery. She was small for gestational age (below the third percentile: weight 1.375 g, height 35 cm, and cephalic circumference 24.5 cm) and the pla-centa weighed 325 g (placental coefficient: 0.236; reference value: 0.182 ± 0.023). The newborn physical examination showed a flat midface, low nasal bridge and short nose, overlapping sutures and redundant occipital skin folds, asymmetrical microphthalmia, upper and lower limb contractures, and valgus deformities of the knees. A cranial computed tomography scan revealed cerebral atrophy with the partial collapse of the skull (**Figure 1A**), ven-triculomegaly, supratentorial hydrocephalus, ocular globe asym-metry and multiple intracranial periventricular calcifications (**Figure 1B**), along with intraocular calcifications (**Figure 1C**). Indirect ophthalmoscopy revealed atrophy at all quadrants, including the optic nerve in the right eye, and atrophy and peripheral pigmentation in the upper and posterior poles of the

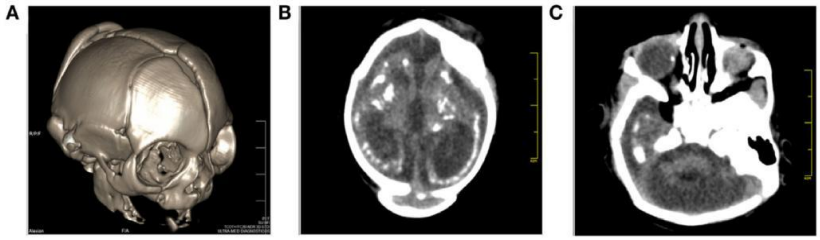

Figure 1 | Cranial computed tomography (CT) images of the baby born after Zika virus infection in pregnancy. **(A)** Sagittal localizer CT image of the markedly abnormal skull shape, **(B)** axial CT images showing microcephaly, cerebral atrophy, and multiple dense intracranial periventricular calcifications located in the subcortical white matter at the gray matter–white matter interface, and **(C)** ocular calcification.

retina in the left eye. The echocardiographic evaluation showed a patent foramen ovale. A qPCR test for ZIKV in a liquor sample taken at birth yielded inconclusive results (data not shown). At ages of 2 and 4 months, the infant had an undetectable HIV viral load. When the child was 6 months of age, the mother's serum tested IgG-positive and IgM-negative for ZIKV and negative for anti-Dengue and anti-Chikungunya viral antibodies (Table S1 in Supplementary Material) . The timeline of events and findings is presented in Figure S1 in Supplementary Material.

Ethical Procedures

All procedures performed during this work were approved by the Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ (CAEE: 65924217.4.0000.5248). The mother of the index patient gave written consent and permission for the publication of data and images.

Sample Collection

At delivery, samples from the index case placenta were collected and fixed following various histopathological techniques. Blood samples from the pregnant woman were collected postpartum. A spinal liquor sample was collected from the infant. Samples were collected at the Hospital Plantadores de Cana, Campos dos Goytacazes, RJ, Brazil. As a reference control, a sample of term placenta from a healthy donor was included.

Histopathological Analysis

Samples from the placentas were fixed in formalin (10%), dehydrated in ethanol, clarified in xylene and blocked in paraffin resin. Tissue sections were cut (4 µm thick), deparaffinized in three baths of xylene and rehydrated with decreasing concentrations of ethanol (100 to 70%). Sections were stained with hematoxylin and eosin for histological examination and with periodic acid–Schiff to examine extracellular matrix (EM) morphometry. Stained specimens were visualized by light microscopy (Olympus, Tokyo, Japan), and digital images were obtained using Image-Pro Plus software version 4.5. The case and control samples were coded and handled in a blind test.

Morphometry and Statistical Analysis

The slides were visualized under a light microscope (Olympus), and the EM area was analyzed using Image-Pro Plus software version 4.5. Fifty fields were randomly acquired at 400× magnification from both placentas (Zika-infected and control). The regions stained with PAS were measured, and the percentage of EM was calculated (histological feature/total area of the image). Data were analyzed with GraphPad Prism software v 6.0 (GraphPad Software, CA, USA) using non-parametric statistical tests. Significant differences between the analyzed groups were determined using the Mann–Whitney test with a threshold of $P < 0.05$.

Immunohistochemistry Procedures

The paraffin-embedded tissues were cut (4 µm), deparaffinized in xylene and rehydrated with alcohol. Antigen retrieval was performed by heating the tissue in the presence of citrate buffer. Next, tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in Tris–HCl (pH 7.4). Sections were incubated in Protein Blocker solution (Spring Bioscience, CA, USA) for 5 min at room temperature to reduce non-specific binding. They were then incubated overnight at 4°C with a 1:200 dilution of the mouse monoclonal antibody 4G2 (16), which is specific for the fusion loop at the extremity of domain II of the Flavivirus group antigen envelope (E) protein (16), or the mouse monoclonal IgG antibody against Zika virus non-structural protein NS1 (Arigo Biolaboratories, Taiwan, Republic of China), or with a 1:40 dilution of the mouse mono-clonal antibody specific for the p24 protein of HIV (Agilent— Dako, CA, USA). The next day, sections were incubated with a rabbit anti-mouse IgG conjugated to horseradish peroxidase (Spring Bioscience Corporation, CA, USA) for 40 min at room temperature. Reactions were revealed with diaminobenzidine (Agilent) as a chromogen, and the sections were counterstained with Meyer's hematoxylin (Agilent).

Immunofluorescence Assay

The paraffin-embedded tissues were processed as above, except for incubation with 1% bovine serum albumin for 30 min, and permeabilized with 0.5% Triton X-100 at room temperature. Slides were co-stained overnight at 4°C with a 1:200 dilution of a mouse monoclonal antihuman proliferating cell nuclear antigen (PCNA) IgG (ThermoFisher, OK, USA) or a mouse monoclonal anti-Zika NS1 IgG, and a rabbit monoclonal antihuman CD11b IgG (Abcam, Cambridge, UK); or with a 1:40 dilution of the mouse monoclonal antibody specific for the p24 protein of HIV (Agilent—Dako, USA). Sections were incubated with Alexa 488-conjugated rabbit anti-mouse IgG, Alexa 555-conjugated goat anti-rabbit IgG, or Alexa 555-conjugated goat anti-mouse IgG (ThermoFisher). Slides were analyzed using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany).

Electron Microscopy Procedures

Tissue samples were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2), post-fixed with 1% buffered osmium tetroxide, dehydrated in an acetone series (30, 50, 70, 90, and 100%), and embedded in EPON polymerized at 60°C for 3 days. Ultrathin sections (60–90 nm) were contrasted with uranyl acetate and lead citrate and were visualized using a JEOL 1001 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

RESULTS

Histopathological analysis of the ZIKV-infected placenta revealed severe damage to the maternal decidua and CV. In the control placenta, we observed a regular structure of decidual parenchyma with typical decidual cells (DC) and capillaries exhibiting regular endothelial cells. Likewise, CV showed normal STB, CTB, and endothelial cells (**Figures 2A–C**). The most prominent lesion in the placenta from the Zika -infected patient was the presence of large and diffuse areas of fibrinoid necrosis (FN) in the maternal decidua (**Figures 2D,E,G,H**). The maternal portions of the placenta also presented diffuse edema, fibrosis, vascular endothelial thickening, degeneration, vascular congestion (VC) and focal areas of mononuclear or

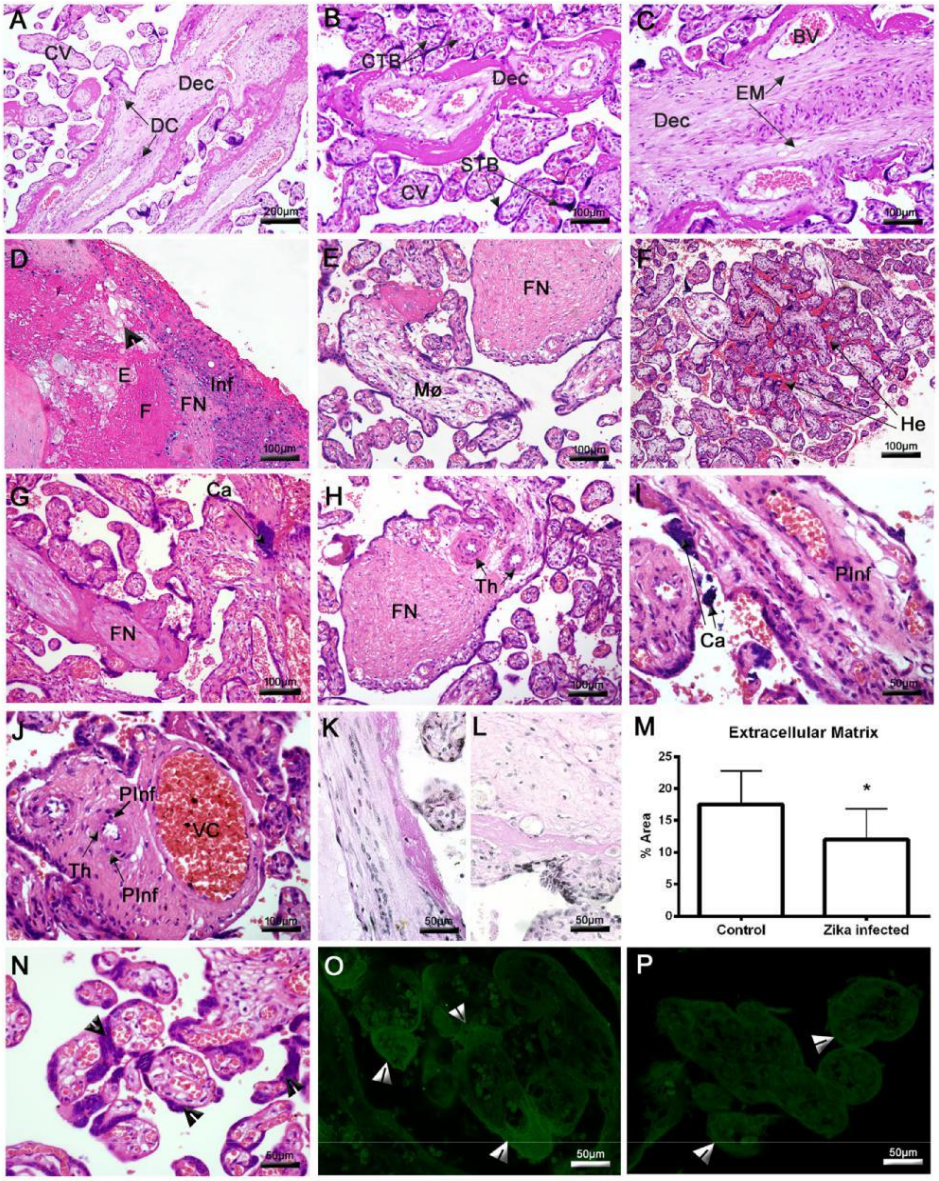

Figure 2 | Histopathological analysis of the placenta. **(A–C)** Placenta of a non-ZIKV patient stained with H&E and presenting normal features: maternal decidua (Dec), decidual cells (DC), chorionic villi (CV), syncytiotrophoblasts (STB), cytotrophoblasts (CTB), extracellular matrix (EM), and blood vessels (BV). **(D–J,N)** Sections of ZIKV-infected placental tissue stained with H&E, showing abnormalities in the decidua, including edema **(E)**, fibrosis **(F)**, fibrinoid necrosis (FN), mononuclear inflammatory infiltrate (Inf), macrophages (Mø), endothelial thickening (Th), cellular degeneration [arrowhead in panel **(D)**], calcification (Ca), and other pathological features in CV, such as perivascular inflammatory infiltrate (PInf), vascular congestion (VC), hemorrhage (He), and inordinate proliferative STB [arrowheads in panel **(N)**]. **(K)** Placental sections from a non-ZIKV and **(L)** a ZIKV-infected patient stained with PAS, evidencing highlighting the EM. **(M)** The percent EM area was quantified in both cases; asterisks indicate statistically significant differences between samples: $*P < 0.05$. **(O)** Immunofluorescence analysis of proliferating cell nuclear antigen expression in the STB of a ZIKV-infected and **(P)** a non-ZIKV patient.

perivascular inflammatory infiltrates (**Figures 2D,E,G–J**). The decidua showed dense and heterogeneous calcification, which can be consistent with third-trimester gestation (**Figures 2G,I**). Since the histopathological analysis indicated degeneration of the decidua, the EM was assessed using PAS staining. Morphological analysis revealed a significant reduction in the total area of EM in the placenta from the Zika -infected patient (12.02% ± 1.33) compared with the control placenta

(17.52% ± 1.45) (**Figures 2K–M**). An investigation of the CV revealed an area of extensive hemorrhage and prominent STB (**Figures 2F,N**). A higher density of PCNA was observed in the STB of the index placenta compared with the control placenta (**Figures 2O,P**). PCNA acts as a scaffold to recruit proteins involved in DNA replication or repair.

Placentas were tested for the presence of ZIKV antigens using an immunohistochemistry assay. Virus antigens were observed only in samples from the ZIKV-infected patient. No immunostaining was observed in samples from the control placenta (**Figures 3A,D,E**). ZIKV envelope proteins were identi-fied mainly in DC (**Figures 3B,C**), whereas the NS1 protein was detected in the cytoplasm of several placental cells, such as decidual and endothelial cells in the maternal decidua. In the CV, the NS1 protein was detected in CTB, STB, and Hofbauer cells, which strongly suggests that viral replication occurred in these cells (**Figures 3F–H**). Further evidence for ZIKV replication in these cells was provided by the positive immunofluorescence signal for NS1 protein (green fluorescence) in several placental cells and its co-localization with CD11b (red fluorescence), used to identify infected mononuclear cells (**Figure 3J**). As expected, no positive reactions against NS1 were observed in control tissue (**Figure 3I**). The term placenta was negative for HIV-specific p24 protein as assessed by immunohistochemistry and fluorescence (Figure S2 in Supplementary Material).

No evidence of ultrastructural changes was noted in the control patient (**Figures 4A–C**). In contrast, the infected placenta showed various alterations such as thickening of the endothelial basement membrane (**Figure 4D**), the nuclei of the STB with dispersed chromatin aggregated in the vicinity of the nuclear membrane, and rarefied cytoplasm with absent organelles (**Figure 4E**). Moreover, smaller mitochondria and fewer cristae were observed, and the endoplasmic reticulum (ER) exhibited dilated cisterns in CTB (**Figure 4F**). Ultrathin sections of placental tissue allowed the identification of clusters with dense virus -like particles, located in damaged cytoplasmic vesicles of CTB (**Figure 4G**). We also observed these virus-like particles near disrupted areas of the ER. The remains of membranes could be seen at the periphery of these clusters (**Figure 4H**). These particles were approximately 25 nm in diameter, which is consistent with the physical dimensions of ZIKV (**Figure 4I**). We did not observe virus-like particles with diameters consistent with mature or immature HIV (range 110–146 nm).

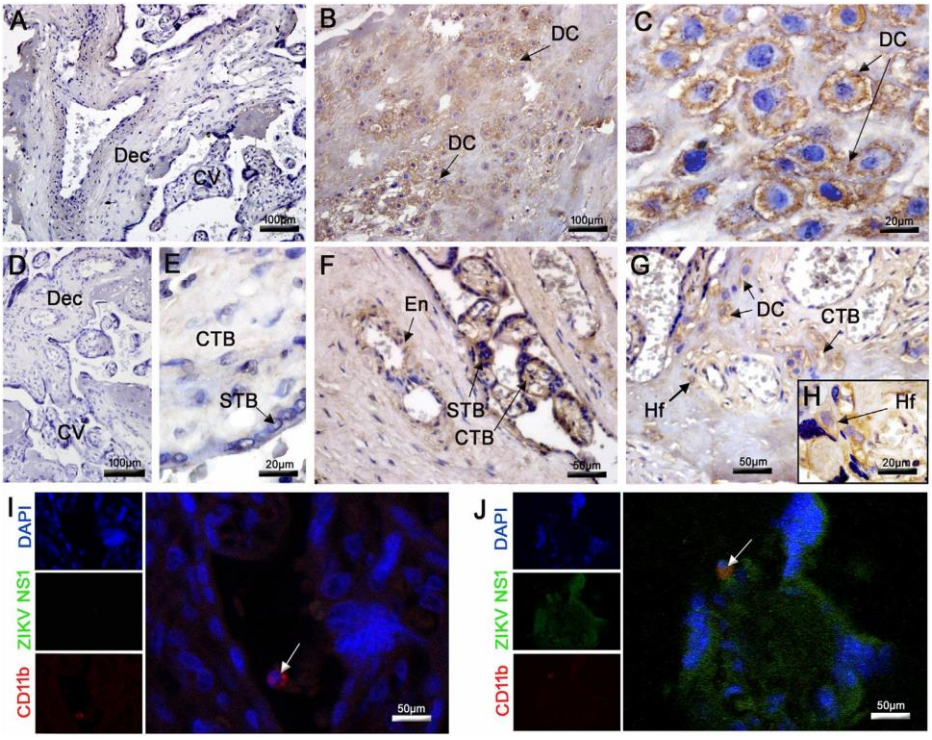

Figure 3 | Detection of ZIKV in the placenta. **(A,D,E)** The E and NS1 antigens of ZIKV were not detected by immunohistochemistry in the control placenta. **(B,C)** Detection of ZIKV E protein in decidual cells (DC) by immunohistochemistry in the infected placenta. **(F–H)** The NS1 protein of ZIKV was also detected by immunochemistry in the endothelium (En), DC, syncytiotrophoblasts (STB), cytotrophoblasts (CTB), and Hofbauer cells (Hf). **(I,J)** Co-localization by immunofluorescence of the NS1 protein (fluorescent green) and CD11b for identification of leukocytes (fluorescent red). Nuclei were stained using DAPI (fluorescent blue). **(I)** ZIKV NS1 antigen was not detected in the control placenta. **(J)** Cells presenting dual staining (green and red) were observed in the ZIKV-infected placenta.

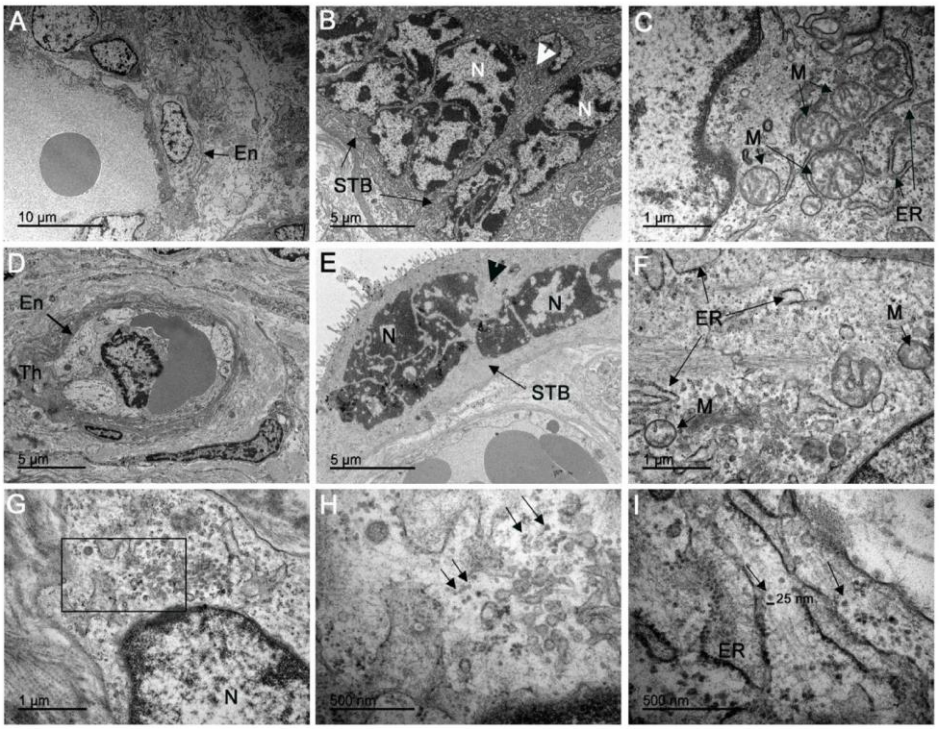

Figure 4 | Electron microscopy analysis of ultrathin placental sections showed virus-like particles. **(A–C)** Electron microscopy of ultrathin sections of one non-ZIKV case exhibited regular endothelial cells (En), syncytiotrophoblasts (STB), and CTB organelles, such as mitochondria (M) and endoplasmic reticulum (ER). **(D–F)** Electron micrographs of ZIKV-infected placenta showing thickening of the basement membrane (Th) of the endothelium (En), dispersed chromatin in syncytiotrophoblast nuclei (N) gathered in the vicinity of the nuclear membrane, and rarefied cytoplasm with absent organelles (arrowhead); mitochondria (M) in cytotrophoblasts were smaller, with fewer cristae, and the ER exhibited dilated cisterns. **(G)** Cytotrophoblast of a ZIKV-infected patient with a cluster of virions in the cytoplasm. **(H)** In the same area, at a higher magnitude, we observed these virus-like particles located near disrupted ER. **(I)** Measurement using the scale bar showed that the particles have a diameter of approximately 25 nm, consistent with ZIKV.

DISCUSSION

Overall, the clinical and placental evaluations support a diagnosis of intrauterine infection with ZIKV and exposure to HIV infec-tion, with an outcome of severe congenital Zika syndrome in an HIV-exposed uninfected infant. Similar structural abnormali-ties of the skull and brain have been documented in presumed and confirmed ZIKV infection in pediatric HIV-unexposed cohorts (17, 18). We note that the progression of the fetal disease to microcephaly occurred within a period of 7 weeks in the second trimester, a timeframe that is consistent with the maternal perfusion of the placenta occurring in the late first to the second trimester. We speculate that the ZIKV infection of the fetus occurred early in the second trimester because the extent of the sequelae did not include abnormalities otherwise related to first-trimester development (i.e., holoprosencephaly and craniosynostosis) (18). ZIKV RNA and ZIKV antigens have been detected in several types of placental cells from pregnancies with histopathologic alterations by ZIKV infection and localized ZIKV antigens or RNA in placental all cells (12, 19). Furthermore, published data indicate that infection with another flavivirus, such as Dengue, is associated with a transient decrease in HIV viral load, and no severe Dengue or HIV progression is observed during coinfection (20). This is consistent with our observations of the current HIV-positive pregnant patient infected by ZIKV. Notwithstanding the histopathological damage associated with the ZIKV infection of the placenta and the detectable viral load of HIV in the pregnant woman, the HIV did not infect the placenta. Importantly, a recent study correlating the placental characteris-tics from pregnancies in HIV-infected-positive and HIV-negative women described no specific lesions in infected placentas (21). Moreover, the birth head circumference of the newborns was similar in the two groups. In addition, vertical transmission was avoided by the use of antiretroviral drugs (21).

Notwithstanding the extent and severity of the gross damage caused to both the placenta and the fetal brain by the ZIKV infec-tion, the severe intrauterine growth deficiency was compatible with life. In cases of maternal coinfection (i.e., Cytomegalovirus and HIV coinfection), in which there is a considerable inflamma-tory response in the placenta, often one viral infection predisposes the patient to a higher risk of acquiring the other (22). In a recent prospective cohort study in 134 ZIKV-positive/HIV-negative

pregnant women with new-onset rash manifestations, neither the severity of maternal ZIKV disease, the virus RNA load nor the prior existence of Dengue antibodies was significantly associated with abnormal birth outcomes (23). We cannot rule out a col-lateral damage effect from the HIV co-exposure on the placental histopathological abnormalities caused by ZIKV infection.

The findings in the abnormally large term placenta suggest that ZIKV spread from the maternal decidua to the CV and reached the fetus by replication and transmission from cell to cell. This hypothesis is based on the detection of NS1 protein in the cyto-plasm of several placental cells, such as decidual and endothelial cells in the maternal decidua, and CTB, STB, and Hofbauer cells, which supports the hypothesis of active viral replication. The occurrence of membranous clusters of viral particles in the cytoplasm of CTB is therefore reminiscent of replication factories. Moreover, the observed enrichment with the PCNA expression signal (a protein involved in DNA replication and repair) (24) in the STB indicates an attempt by the cells to repair the injured DNA. Some studies showed that the binding of PCNA to repair proteins is direct, and that the inhibition of its expression impairs the mechanism of DNA repair (25, 26). Tissue alterations such as FN, edema, calcification, and mononuclear inflammatory infil-trates are commonly seen in ZIKV-infected premature placentas (9, 12, 18). Significantly, diffuse fibrosis, vascular endothelial thickening, cellular degeneration, hemorrhage, and VC had not been previously reported in ZIKV-infected term placentas. Furthermore, we observed infected mononuclear cells that are capable of supporting replicating virus, including dendritic cells and macrophages (Hofbauer cells), which are known to be primary targets of another flavivirus, Dengue (27). However, an *in vitro* study with cells isolated from mature placenta showed that placental macrophages and CTB are permissive to ZIKV infection (14). In addition, Tabata et al. (13) observed that all placental cells, as well as immune cells, in the tissue present receptors capable of binding to the virus and mediating cell entry by endocytosis.

CONCLUDING REMARKS

It is important to note that the viral antigens persisted in the placental tissue months after the infection occurred. Ultimately, the presence of dense, virus-like particles consistent with the size of ZIKV, seen near the ER of CTB, was the most definitive indication of a persistent viral infection in placenta, from the second trimester until delivery. Our results confirm that maternal ZIKV infection during pregnancy can result in placental and fetal injury. Furthermore, in countries where the prevalence of HIV-positive pregnant women is high, such as in Sub-Saharan African nations (5.3% average prevalence), more cases of severe Zika disease in HIV-exposed fetuses are expected to occur if the epidemic potential of ZIKV increases there (28). The present index case serves as evidence for the importance of preparedness.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ (CAEE: 65924217.4.0000.5248)" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ."

AUTHOR CONTRIBUTIONS

KR, MP, RF, and EM-A designed the study. LS, CB-d-O, TS, and RF collected samples and performed clinical and tomography exams. KR performed all research experiments for placental evaluation. FS, PN, EA, NS, and GT optimized or supported immunohistochemical experiments. KR and EM-A wrote the manuscript. KR, MP, CB-d-O, and JC analyzed the experimental results. MP, JC, RF, and TS edited the manuscript. All the authors gave final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fimmu.2017.01704/full#supplementary-material.

Figure S1 | Timeline of relevant events, studies and findings in the presented case of intrauterine co-exposure to ZIKV and human immunodeficiency virus (HIV).

Figure S2 | Immunohistochemistry and fluorescence for p24 human immunodeficiency virus (HIV) protein. Tissue sections were deparaffinized and rehydrated, and antigen retrieval was performed by heating the tissue in the presence of citrate buffer. Sections were blocked and then incubated overnight at 4°C with a 1:40 dilution of the mouse monoclonal antibody specific for the p24 protein of HIV. The next day, sections were incubated with a rabbit anti-mouse IgG-HRP conjugate for immunohistochemistry or Alexa 555-conjugated goat anti-mouse IgG for immunofluorescence. Reactions were revealed with diaminobenzidine as the chromogen and the sections were counterstained with Meyer's hematoxylin for immunohistochemistry. **(A,C)** The p24 antigen of HIV was not detected by immunohistochemistry or by immunofluorescence in the control placenta. **(B,D)** The p24 antigen of HIV was not detected by immunohistochemistry or by immunofluorescence in the ZIKV index case placenta.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Material suplementar do Artigo 1

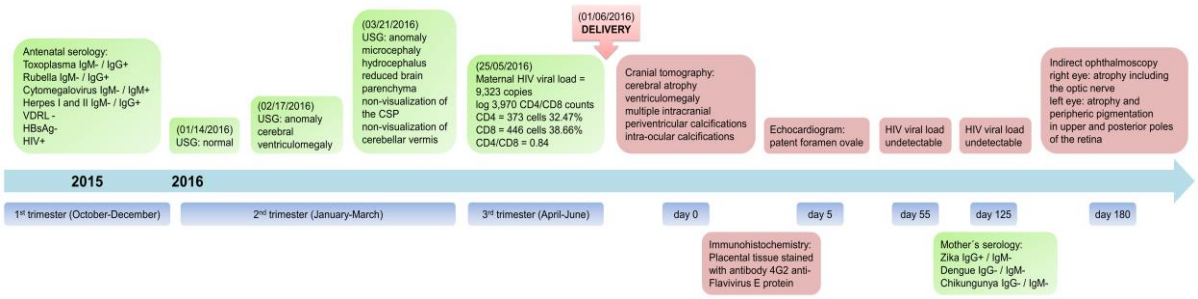
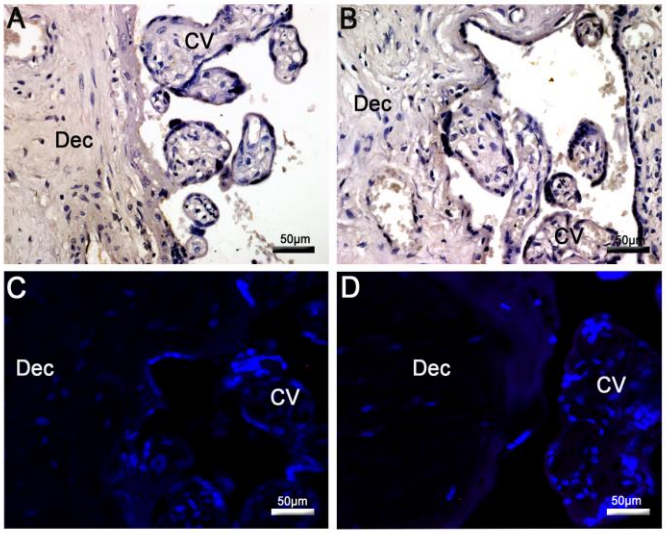


Figure S1. *Timeline of relevant events, studies and findings in the presented case of intrauterine co-exposure to ZIKV and human immunodeficiency virus (HIV)*



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Supplementary Table 1. Serology results of the index case.

	Antenatal serology (mother) [a]		**Baby girl** [b]		
Test	**Values**	**Result**	**Values**	**Result**	**Reference values**
Anti-Cytomegalovirus IgG	171.8 UA/mL	Positive	49.6 UA/mL	Positive	<6 UA/mL
Anti-Rubella IgG	84.3 UI/mL	Positive	13.2 UI/mL	Positive	>10 UI/mL
Anti-Toxoplasmosis IgG	163.7 UI/mL	Positive	19.6 UI/mL	Positive	>3 UI/mL
Anti-Herpes I and II IgG	24.6	Positive	6.7	Positive	>1,25
Anti-Cytomegalovirus IgM	0.12	Negative	0.77	Negative	<0.85
Anti-Rubella IgM	0.18	Negative	0.06	Negative	<1.20
Anti-Toxoplasmosis IgM	0.20	Negative	0.29	Negative	<0.5
Anti-Herpes I and II IgM	Non-reactive	Negative	Non-reactive	Negative	<0.75
VDRL	Weakly reactive	Negative		ND[d]	
Anti-HIV 1 e 2	88 nm	Positive		Negative	>1

	Postnatal serology (mother) [c]		
Test	**Values**	**Result**	**Reference values**
Anti-Zika virus IgG	6 UR/mL	Positive	>1,09
Anti-Dengue virus IgG	Non-reactive	Negative	
Anti-Chikungunya virus IgG	<0.8	Negative	<0.8
Anti-Zika virus IgM	0.04	Negative	<0.8
Anti-Dengue IgM	Non-reactive	Negative	
Anti-Chikungunya IgM	Non-reactive	Negative	

[a] during the 1st trimester
[b] at two months of age
[c] six months after delivery
[d] not performed

2.2 **Placental inflammation and fetal injury in a rare Zika case associated with Guillain-Barré syndrome and abortion (Artigo publicado)**

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Placental Inflammation and Fetal Injury in a Rare Zika Case Associated With Guillain-Barré Syndrome and Abortion

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Zika virus (ZIKV) is an emerging virus involved in recent outbreaks in Brazil. The association between the virus and Guillain-Barré syndrome (GBS) or congenital disorders has raised a worldwide concern. In this work, we investigated a rare Zika case, which was associated with GBS and spontaneous retained abortion. Using specific anti-ZIKV staining, the virus was identified in placenta (mainly in Hofbauer cells) and in several fetal tissues, such as brain, lungs, kidneys, skin and liver. Histological analyses of the placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema and necrosis in placenta, as well as tissue disorganization in the fetus. Increased cellularity (Hofbauer cells and $TCD8^+$ lymphocytes), expression of local pro-inflammatory cytokines such as IFN-γ and TNF-α, and other markers, such as RANTES/CCL5 and VEGFR2, supported placental inflammation and dysfunction. The commitment of the maternal-fetal link in association with fetal damage gave rise to a discussion regarding the influence of the maternal immunity toward the fetal development. Findings presented in this work may help understanding the ZIKV immunopathogenesis under the rare contexts of spontaneous abortions in association with GBS.

Keywords: Zika virus, immune response, Guillain-Barré syndrome, fetal infection, histopathology

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INTRODUCTION

Zika virus (ZIKV) is an emerging mosquito-borne pathogen that belongs to the *Spondweni* serocomplex of the *Flavivirus* genus, Flaviviridae family (Didier and Gublerb, 2016). Zika fever emerged in Latin America in 2015–2016 and rapidly became a worldwide public health concern (Singh et al., 2016; Slavov et al., 2016). This massive outbreak highlighted possible correlations between the infection and dangerous complications such as Guillain-Barré syndrome (GBS) (Araujo et al., 2016; Malkki, 2016) and congenital microcephaly (Araujo et al., 2016; Garcez et al., 2016). In the absence of specific treatment or vaccine, only in Brazil the number of accumulated

infections was estimated between 440,000 and 1,300,000 (Bogoch et al., 2016), with a prevalence of microcephaly of nearly 100 cases per 100,000 live births (Ventura et al., 2016). As an outcome, several unanswered questions, especially regarding the circumstances that may explain a possible connection between the infection and these complications, became a relevant matter of debate.

Initial attempts to model the vertical ZIKV transmission included investigations using immunocompetent mice (Cugola et al., 2016; Vermillion et al., 2017). As these animals are in general resistant to the infection due to virus inability to circumvent the interferon-α/β response (Grant et al., 2016), these models are limited in providing mechanistic explanations to describe pathogenesis. In alternative approaches, genetically modified animals, which are knockout for interferon receptors (IFNARs) or downstream signaling targets, such as IRF3 and IRF7, were employed for investigation (Miner et al., 2016; Yockey et al., 2016). In these reports, despite the characterization of injuries in the fetal brain and the viral escape through the trans-placental route, the animal immunological restriction limited the comprehension of the host response upon infection. In a recent report, an animal model of ZIKV infection involving pregnant non-human primates revealed that, upon prolonged viremia, several fetal tissues, as well as the maternal-fetal interface, were affected (Nguyen et al., 2017). Subjects were found to respond to ZIKV with proliferation of $CD16^+$ NK cells and $CD8^+$ effector T cells. In addition, the maternal-fetal interface was marked by suppurative placentitis and deciduitis with variable mineralization and necrosis. While reports based on ZIKV-infected non-human primates are valuable due to deep similarities between macaques and human pregnancies, for a better description of the immunopathological events and their impact toward the maternal-fetal link it would be ideal to explore human case samples.

Based on the above considerations, here we investigated a rare Zika case, in which an infected 28-year-old pregnant patient was diagnosed with GBS and had a spontaneous abortion.

BACKGROUND

Clinical Presentation

All procedures in this work were approved by the FIOCRUZ Ethics Committee for studies with Zika case and control (CAEE: 65924217.4.0000.5248). The legal representative (mother) of the involved patient provided written consent for the publication of data.

A 28-year-old woman, pregnant, black, housekeeper, from São Francisco do Itabapoana, RJ, was admitted to the hospital on June 29th 2016, presenting weakness in the lower limbs and a consequent inability to ambulate for the previous week. The patient affirmed not having experienced similar episodes previously and claimed to be free of comorbidities. The patient reported rash on her limbs, pruritus and vomiting approximately 1 month before admission to the hospital. Initial obstetric examinations showed globular abdomen, unlimited uterus and absence of vaginal bleeding. The fetal heartbeat was not detected in the Sonar Doppler. Transvaginal ultrasonography evidenced a single fetus with longitudinal status and cephalic circumference suggesting 15 weeks of gestational age, normohydramnios, lack of heartbeat, and non-apparent active movements, which lead to the diagnosis of death and retained fetus (stillbirth). The patient was admitted to curettage and remained in hospital for 30 days. Analysis of the cerebrospinal fluid obtained by lumbar puncture and exams of the patient's peripheral blood revealed normal parameters (Table S1). The IgM serology for Dengue, Chikungunya, Zika, Epstein Barr and Cytomegalovirus were non-reactive (in further sections of this paper, diagnosis of ZIKV infection was confirmed by specific staining in placental and fetal tissues). The patient evolved to ascending and symmetrical flaccid tetraparesis, paresthesia, areflexia, presenting hands with pendular movement, dysautonomia (resting tachycardia and hypertension) and signs of respiratory insufficiency (mild dyspnea at rest which worsens upon effort), characterizing the GBS. The patient was further treated with intravenous immunoglobulin (30 g/day) for five days, atenolol 50 mg 12/12 h, amlodipine 5 mg 24 h, motor rehabilitation, respiratory physiotherapy and psychological intervention. In the second week of disease evolution, the neurological examination showed symmetric flaccid tetraparesis, motor incoordination, global areflexia and sensitive disorders (tactile and thermal distal hypoesthesia). Neurological follow up during and after the hospitalization period are described in Table S2. During the fourth week of disease progress, the patient was admitted to electroneuromyography, which revealed peripheral, acquired, chronic, symmetrical, diffuse, myelin and axonal (predominantly myelin), sensory and motor polyneuropathy: a condition that affected the peripheral nerves of the upper and lower limbs. In addition to that, the patient showed signs of neurogenic myopathy and muscular denervation. Together, these findings were compatible with polyradiculoneuropathy. By the end of the fourth week, the patient was discharged from the hospital under prescription of antihypertensive drugs and still being followed up by motor physical therapy. After 40 days of hospital discharge, the patient was orthostatic, walking-dependent for small distances and with pain sequels in the lower limbs. Five months later, residual sequels were still present with autonomous difficult scarvant gait. After 12 months, the gait patterns were regular; however, the speed and execution of upward and downward movements remained affected. Finally, the patient returned to her daily activities. Methods performed in this work using the placenta and fetal organs are described in Supplementary Material.

RESULTS

Investigation of the Placental Tissue

ZIKV Infection Leads to Histopathological Damage in Placenta

The histopathological analysis considering the patient's placenta showed relevant damage in the membrane, maternal decidua and chorionic villi. We can highlight large areas of hemorrhage, diffuse fibrinoid necrosis and inflammatory infiltrates formed by mononuclear cells. In addition, regions with

cell degeneration and macrophages with clear cytoplasm in membrane and chorionic villi, decidual edema and macrophages were also found. We also noted a decrease in blood vessels of chorionic villi. The decidual region showed dense calcification, which is commonly observed only during the third trimester of gestation (**Figures 1D–K**). As expected, control samples showed regular arrangement of decidual parenchyma. Controls also exhibited normal chorionic villi,

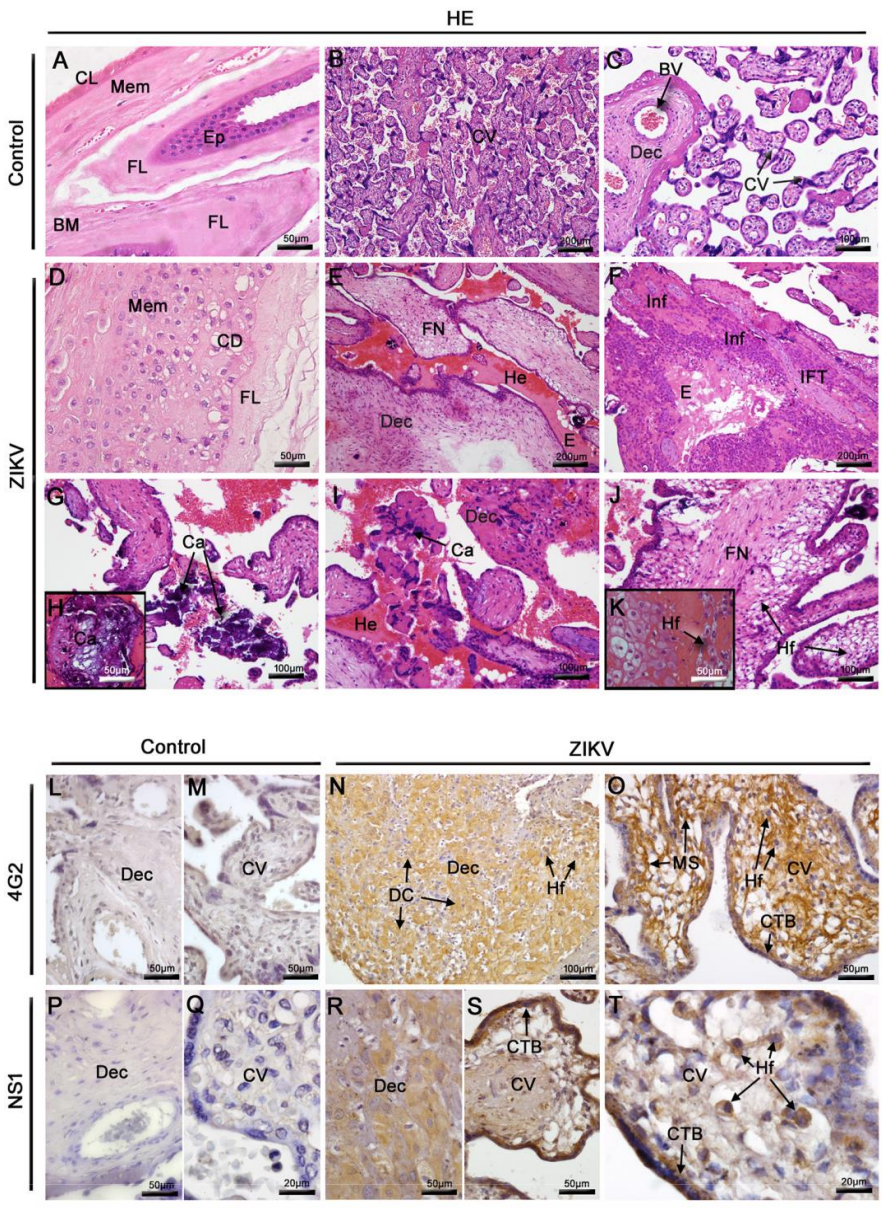

FIGURE 1 | Histopathological analysis of the placenta and detection of ZIKV. **(A–C)** Placenta of a non-ZIKV patient stained with H.E. and presenting normal features: membrane (Mem), (MB) basal membrane, (FL) fibroblastic layer, (CL) compact layer, (Ep) epithelium, chorionic villi (CV), maternal decidua (Dec), and blood vessels (BV). **(D–K)** Sections of ZIKV-infected placental tissue stained with H.E., showing abnormalities in membrane, with cellular degeneration (CD), in the decidua and chorionic villi, including fibrinoid necrosis (FN), hemorrhage (He), mononuclear inflammatory infiltrate (Inf), infarct (IFT), calcification (Ca) and Hofbauer cells with clear cytoplasm (Hf). **(L,M,P,Q)** The flavivirus E protein and NS1 antigens of ZIKV were not detected by immunohistochemistry in the control placenta. **(N–O)** Detection of ZIKV E protein in decidual cells (DC), cytotrophoblasts (CTB), mesenchymal cells (MS) and Hofbauer cells (Hf) of the infected placenta. **(R–T)** The NS1 protein of ZIKV was also detected by immunohistochemistry in decidual cells (DC), cytotrophoblasts (CTB) and Hofbauer cells (Hf).

syncytiotrophoblasts, cytotrophoblasts and endothelial cells (**Figures 1A–C**).

The patient's placental tissue was screened for the detection of ZIKV NS1 protein and E protein using immunohistochemistry. Of note, the anti-NS1 antibody used in these assays is ZIKV specific, thus, is able to diff erentiate ZIKV from other flaviviruses. While the viral antigens were detected in samples from the aff ected patient, no immunostaining was observed in samples considering the control placenta (**Figures 1L,M,P,Q**). E structural viral proteins were detected in decidual cells of the maternal portion, cytotrophoblasts and mesenchymal cells of chorionic villi (**Figures 1N,O**). In the placental portion toward the fetal side, the NS1 protein was detected in cytotrophoblasts, Hofbauer cells of chorionic villi and also in decidual cells (**Figures 1R–T**). Viral detection occurred mainly within the cytoplasmic region of cells with minor to indistinguishable staining in the nuclear area. This staining pattern strongly suggests that viral replication occurred in these target cells.

Characterization of Cell Subpopulations, Colocalization With Virus and Quantification of Cytokine-Producing Cells

Since the histopathological analysis showed inflammatory infiltrates in both maternal and fetal areas, we proceeded with immunohistochemical characterization of the cell types present in this tissue. For this, we used anti-CD68 antibodies to stain the Hofbauer cells and anti-CD8/ anti-CD4 antibodies for phenotype arriving lymphocytes. Staining with anti-CD68 revealed an increase in hyperplasic Hofbauer cells spread in chorionic villi and decidua basalis (**Figures 2C,D**). While $CD8^+$ cells were found in the same areas (**Figures 2H,I**), $CD4^+$ cells were not detected within the tissue. The control tissue showed low density of positive cells (**Figures 2A,B,F,G**). After quantification considering 50 distinct fields, the numbers of both $CD68^+$ and $CD8^+$ cells were significantly increased (6- and 4-fold, respectively) in the placenta of the Zika patient, when compared to the control (**Figures 2E,J**).

Further evidence for ZIKV infection in specific cell subpopulations was provided by immunofluorescence assay. In this case, $CD11b^+$ cells (red fluorescence, which we considered as the mononuclear cells) costained with anti-ZIKV NS1 signals (green fluorescence) within several areas of the patient's placenta (**Figure 2L**). Under this analysis, ZIKV NS1 was also detected in placental cells that were negative for anti-CD11b. As expected, no positive reactions against NS1 were observed in the control tissue (**Figure 2K**).

To better characterize the inflammatory process in the patient's placenta, we also investigated the local expression of cytokines related to inflammation. Under this approach, we verified the expression of: TNF-α and IFN-γ, due to their well-known participation in a pro-inflammatory context; and VEGFR2 and RANTES/CCL5, since these markers are implicated with altered vascular permeability (Chen et al., 2008; Dalrymple and Mackow, 2012). TNF-α was detected in Hofbauer cells of decidua and chorionic villi (**Figure 2N**), while expression of IFN-γ was found mostly in macrophages of membrane and decidua (**Figure 2Q**). The expression of VEGFR2 was found also in macrophages throughout the placental membrane (**Figure 2T**). The chemokine RANTES/CCL5 was detected mainly in the endothelium and in Hofbauer cells located within the chorionic villi and decidua (**Figure 2X**). Cells expressing all these cytokines were found in the control tissue, but in smaller amounts (**Figures 2M,P,S,V**). The numbers of cells expressing all these considered markers were significantly increased in the Zika patient's placenta, when contrasted with the non-Zika control tissues (**Figures 2O,R,U,Z**).

Investigation of the Fetal Tissues

Histopathological Alterations in Fetal Organs Caused by ZIKV

The histopathological analysis of the brain tissues collected from the Zika patient's fetus revealed diff use areas of edema, disorganization of the cerebral cortex layers, mainly in the layer of polymorphic cells and degenerate nerve fibers (**Figure 3B**). The analysis of the lung tissues revealed several damaged areas with disorganization of the bronchioles architecture associated with focal areas of hyaline membrane. Other alterations in the lungs included regions of septal thickening, increased cellularity, necrosis in the respiratory epithelium accompanied by cell detachments and the presence of mononuclear cell inflammatory infiltrates (**Figure 3D**). The skin from the aff ected fetus presented diff use areas of edema associated with perivascular lymphocytic infiltrate in the dermis region (**Figure 3F**). In the kidneys, some areas of glomeruli ischemia were observed causing loss of the tubule architecture and its degeneration. This observation was associated with focal mononuclear cell infiltrates (**Figure 3H**). Liver fetal samples from the Zika patient exhibited severe parenchyma and circulatory disorganization. In this organ, the most prominent lesions were the cellular and matrix degenerations that were associated with increased numbers of Kupff er cells (**Figure 3J**). As expected, when considering the samples extracted from the control fetus, all the analyzed sites (brain, lungs, skin, kidneys and liver) presented regular structures (**Figures 3A,C,E,G,I**).

Detection of ZIKV Antigens in Fetal Organs

Using IHC technique to investigate the Zika patient's fetus we detected the flaviviral-E and ZIKV-NS1 proteins in microglial cells and neurons within the cerebral cortex of the brain tissue (**Figures 4B,L**). In the lung, these proteins were detected in alveolar macrophages (**Figures 4D,N**), in mononuclear cells of skin (**Figures 4F,P**), in macrophages of the kidneys (**Figures 4H,R**), and, finally, in hepatocytes and Kupff er cells of the liver (**Figures 4J,T**). No E or NS1 immunostaining was observed in samples from the control organs (**Figures 4A,C,E,G,I,K,M,O,Q,S**).

DISCUSSION

The incidence of GBS in Brazil has been evidently increasing after the ZIKV outbreak (Oehler et al., 2014). A recent study

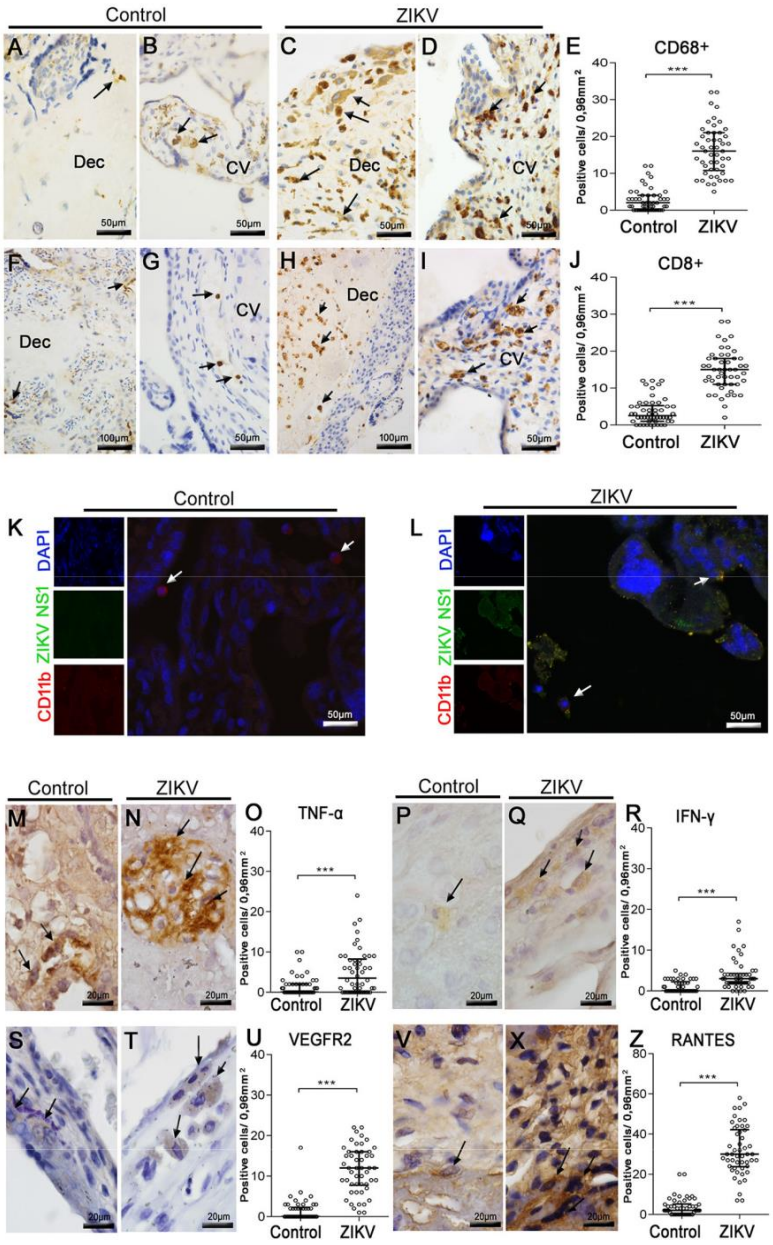

FIGURE 2 | Characterization of mononuclear cell subpopulations in ZIKV-infected placental tissue, colocalization with virus and cytokine-producing cell profile. **(A,B)** Detection of $CD68^+$ cells (Hofbauer cells) by immunohistochemistry in decidua and chorionic villi of control placenta, respectively. **(C,D)** Hofbauer cells in decidua and chorionic villi of ZIKV infected placental tissue, respectively. **(F,G)** Detection of $CD8^+$ cells by immunohistochemistry in decidua and chorionic villi of control placenta, respectively. **(H,I)** $CD8^+$ cells immunostained in decidua and chorionic villi of ZIKV-infected placental tissue, respectively. **(E,J)** Quantification of $CD68^+$ and $CD8^+$ cells in ZIKV case and control, respectively. **(K,L)** Colocalization by immunofluorescence of the NS1 protein (fluorescent green) and CD11b for identification of leukocytes (fluorescent red). Nuclei were stained using DAPI (fluorescent blue). **(K)** ZIKV NS1 antigen was not detected in the control placenta.

(L) Cells presenting dual staining (green and red) were observed in the ZIKV-infected placenta. **(M,N)**. Detection of TNF-α in cells of chorionic villi of control and ZIKV infected placenta by immunohistochemistry, respectively. **(P,Q)** Production of IFN-γ in cells of membrane of control and ZIKV infected placenta, respectively.

(S,T) VEGFR2-expressing cells of decidua in control and ZIKV infected placenta, respectively. **(V,X)** Detection of CCL5/RANTES in cells of chorionic villi of control and ZIKV infected placenta, respectively. **(O,R,U,Z)** Quantification of the number of cells expressing TNF-α, IFN-γ, VEGFR2, and CCL5/RANTES, in ZIKV case and control, respectively. Asterisks indicate differences that are statistically significant between groups (***$p < 0.001$).

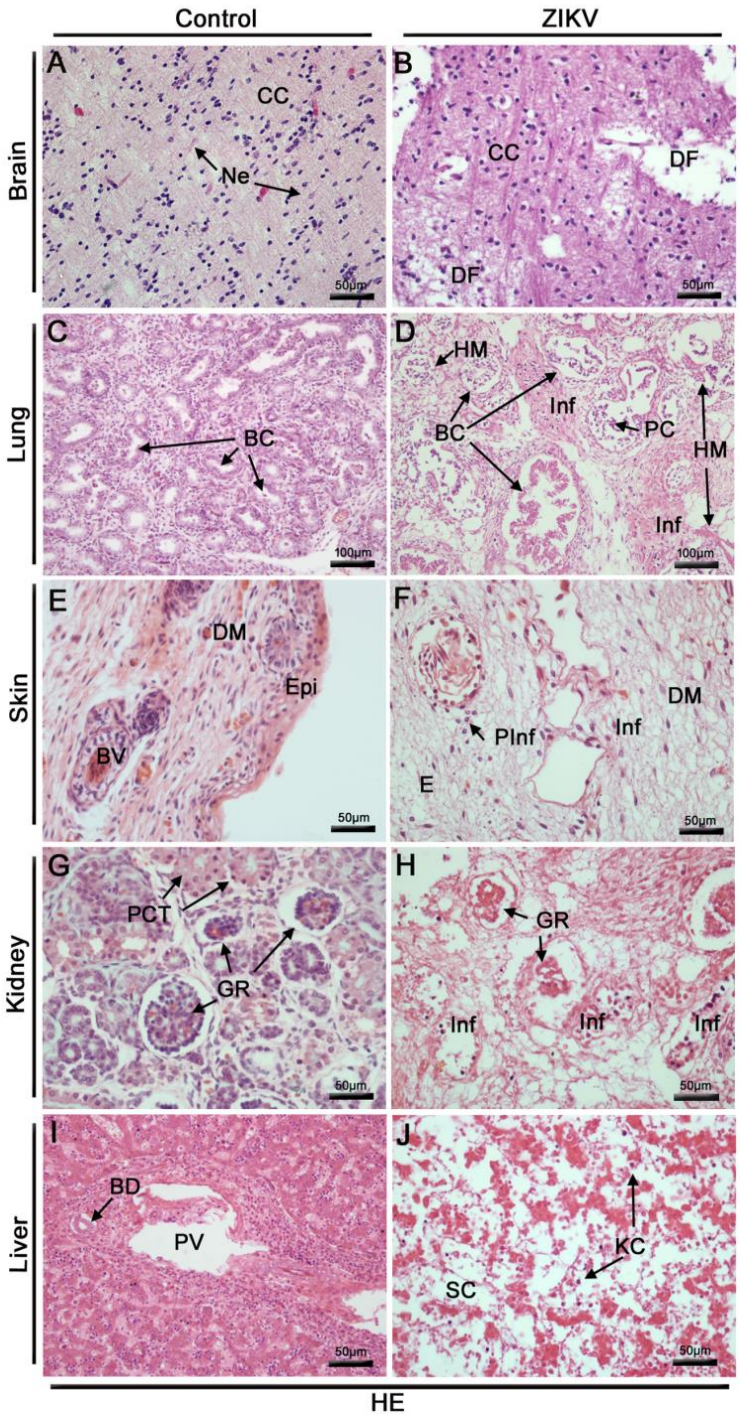

FIGURE 3 | Histopathological analysis of the fetal organs. **(A–J)** All tissues were stained with H. E. **(A)** Brain of a non-ZIKV case presenting normal aspect: cerebral cortex (CC) and neurons (Ne). **(B)** Brain of a ZIKV infected fetus, presenting areas of degenerated nerve fibers (DF). **(C)** Lung section of a control fetus showing normal bronchioles (BC). **(D)** Injuries in fetal lung infected by ZIKV: disorganized bronchioles (DBC) with loss of cylindrical appearance, focal areas of hyaline membrane (HM),

(Continued)

FIGURE 3 | diffuse mononuclear infiltrates (Inf) and peeled cells of respiratory epithelium (PC). **(E)** Skin dermis (DM) of a non-ZIKV case presenting normal aspect, epidermis (Epi), blood vessel (BV). **(F)** Skin dermis of a ZIKV infected fetus, with perivascular and mononuclear infiltrate (PInf and Inf) and areas of edema **(E)**. **(G)** Kidney of a non-ZIKV case presenting normal aspect, with normal glomerulus (GR) and proximal contorted tubules (PCT). **(H)** Kidney sections showing injuries, including: disorganized renal glomerulus (GR), tubular disarrangement and inflammatory infiltrate (Inf). **(I)** Section of a control liver, with normal bile duct (BD) and portal vein (PV). **(J)** Liver of a ZIKV infected fetus, presenting dilatation of sinusoidal capillaries (SC), hepatic parenchymal disorganization and Kupffer cells (KC).

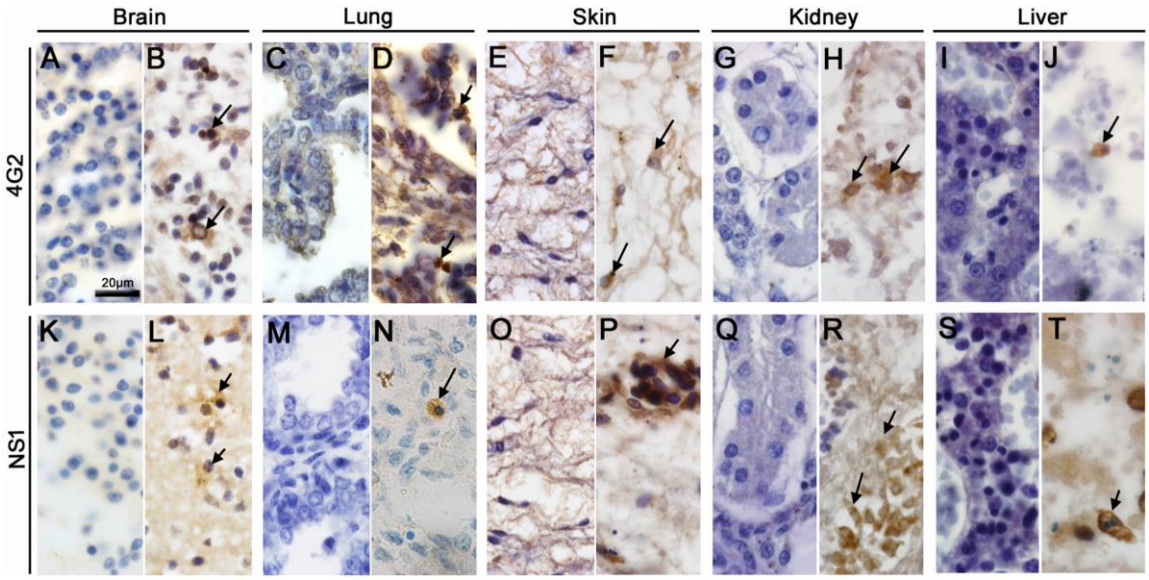

FIGURE 4 | Detection of ZIKV antigens in fetal organs. **(B,D,F,H,J,L,N,P,R,T)** The flaviviral E and ZIKV-NS1 protein were detected in all tissues studied. **(B,L)** Microglial cells and neurons of the brain tissue were positive for ZIKV E protein and NS1, respectively. **(D,N)** ZIKV E and NS1 proteins were detected in alveolar macrophages. **(F,P)** Section of a skin dermis showed mononuclear cells of an inflammatory infiltrate and endothelial cells stained respectively to ZIKV E and NS1 proteins. **(H,R)** ZIKV E protein and NS1 detection in macrophages of the kidney. **(J,T)** Detection of ZIKV E protein and NS1 in hepatocytes, respectively. **(A,C,E,G,I,K,M,O,Q,S)** The E and NS1 antigens of ZIKV were not detected by immunohistochemistry in the control fetus.

showed ZIKV cases with neurological alterations similar to those found in our study, defined as GBS. In these cases, the viremia appeared to persist for longer than normal (Brito Ferreira et al., 2017). While the relationship between Zika fever and GBS still relies solely on epidemiological data, the description of the viral influence toward congenital malformations has become less enigmatic. In this work, we investigated a rare Zika case, which was associated with GBS and spontaneous retained abortion during the 15th week of fetal development. Viral infection was characterized in placental and in several fetal tissues. As found previously (Schaub et al., 2017), this scenario suggested that the case was involved with a high or persistent viremia. Histological analysis of the patient's placenta and fetal organs revealed diff erent types of tissue abnormalities, which included inflammation, hemorrhage, edema and necrosis in placenta and tissue disorganization in the fetus. The patient presented negative IgM serology for several viruses, including ZIKV; however, ZIKV could be detected directly in tissue samples. Based on this, any of the tested viruses (dengue, chikungunya, Epstein-Barr, and Human Citomegalovirus) could also potentially be contributing to the outcome. Nonetheless, given the peculiarity of the clinical presentation, the epidemiological aspect involved, and obviously, the characterization of ZIKV in several areas, we believe that ZIKV may have contributed as the major component for the observed alterations. Of note, the present case happened in an area that had no incidence of yellow fever, which also reduces a probable influence of this disease in the observed results.

In the particular case of the observed placental alterations, one fact that drew our attention was the local increase of numbers of Hofbauer cells in association with the viral infection and pro-inflammatory cytokine production. Infection of Hofbauer cells during the antenatal period not only reflects the critical failure of the protective arrangement, but also highlights a potential pathway for ZIKV vertical transmission. In fact, several research groups have demonstrated either histologically (Noronha et al., 2016; Rosenberg et al., 2017; Rabelo et al., 2017) or by isolated cultures/explants (Jurado et al., 2016; Quicke et al., 2016; Tabata et al., 2016) that these placental macrophages are highly permissive to ZIKV replication. This observation matches the findings from Rosenberg and colleagues, that in a histological study of a Zika case also detected proliferation and hyperplasia

of such resident placental cells (Rosenberg et al., 2017). Another indication that ZIKV-infected placental cells were targeted by immune activation was the detection of $TCD8^+$ cell infiltrates within the tissue. As broadly investigated previously, TCD8-mediated cellular immunity is apparently critical for host's defense against ZIKV infection (Huang et al., 2017; Ngono et al., 2017; Pardy et al., 2017; Wen et al., 2017). Since we found elevated expression of local pro-inflammatory cytokines (IFN-γ and TNF-α), one hypothesis to explain this scenario is that ZIKV-infected Hofbauer cells may have contributed to the establishment of a chemotactic environment for the arrival of specific lymphocytes.

The considerations exposed above gave rise to a little explored discussion when considering the maternal-fetal link under a viral influence: the maternal immune activation (MIA). Initial thoughts considered pregnancy as a temporary immunosuppressed condition that would be necessary to allow a successful fetal development (Medawar, 1948; Silasi et al., 2015). Nowadays, MIA is thought to be a complex process that changes in a dynamical fashion as the pregnancy evolves (Mor and Cardenas, 2010; Racicot et al., 2014; Silasi et al., 2015). Hypothetically, this entire process culminates in a maternal environment designed to sustain and to protect the pregnancy. In general, when the placenta is targeted by viruses, this organ presents an outstanding capacity to hold back infection, and consequently, to prevent the virus from spreading toward the developing fetus (Romero et al., 2007; Cardenas et al., 2010; Ouyang et al., 2014; Bayer et al., 2015). Conversely, what we saw in the Zika case exposed in this work is much closer to an exception of the above outlook. Due to a yet unknown mechanism, ZIKV seems to hold a unique capacity to circumvent MIA and therefore promote relevant infection and inflammation throughout the placental tissue. Considering the patient's placental conditions, we hypothesize that this fact probably created a bridge between the maternal infection and the eff ects observed in the developing fetus.

The patient's placental dysfunction caused by ZIKV infection, given the local inflammation and possible altered vascular permeability (as evidenced by the over-expression of RANTES/CCL5 and VEGFR2), may have impaired the normal balance of this hormonal distribution and consequently negatively contributed to fetal development. Other authors have also proposed that placental and decidual inflammation by ZIKV, or other viruses, would critically impact in the normal development of the fetus (Silasi et al., 2015; Mor, 2016). The overexpression of VEGFRs has previously been associated with pathophysiological damage in placentae, while RANTES expression has been found in large quantities in the acute phase of ZIKV infection (Tsatsaris et al., 2003; Tappe et al., 2016). In this sense, a new assumption is proposed for the circumstances by which ZIKV is able to break through the biological placental barrier and to debilitate the pregnancy as a whole.

Although the fetal control tissue samples were at 15 weeks of embryological development and the organs were not yet mature, we observed large histopathological diff erences between these and tissue samples infected by ZIKV. These injuries are probably due to prolonged viremia in the mother, leading to GBS, fetal involvement and consequently retained abortion. IHC analysis of the patient's fetal brain revealed that this organ was targeted by ZIKV infection, in special the microglial cell types. Several other reports showed that the developing fetal central nervous system (CNS) is highly permissive to ZIKV replication (Kuivanen et al., 2017; Lin et al., 2017; Qian et al., 2017; Rosenfeld et al., 2017). However, despite the well-known tropism of ZIKV to the developing CNS, our findings showed that in the peculiar case of the studied fetus, the infection went beyond the cerebral structure and was found in several peripheral tissues. The commitment of other fetal sites such as the lungs, skin, kidneys and liver supported an idea that the patient was under high or persistent viremia. At the same time, this observation highlights the possibility of novel target tissues when considering an extreme situation, as noted by the clinical features of the studied patient.

CONCLUDING REMARKS

This work describes placental and fetal abnormalities found in a rare Zika case involved with GBS and spontaneous abortion. The clinical scenario gave rise to a novel discussion regarding the influence of maternal immunity toward fetal development. Given the unrecognized prevalence of such an uncommon clinical presentation, samples used in this work are valuable for studying the parallel between the infection and the occurrence of GBS and abortion. Findings from this work may add to the current description of ZIKV congenital pathogenesis.

AUTHOR CONTRIBUTIONS

KR, MP, and JC designed the study; LJS, ML, PS, FR, and LS collected samples and performed clinical exams; KR, NS, and RBO performed all research experiments for placental and fetal evaluation; KR and EO wrote the manuscript; KR, MP, and JC analyzed the experimental results. All authors gave final approval in the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01018/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Material and Methods

Ethics and sample collection

All procedures performed during this work were approved by the Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ for studies with Zika case and control (CAEE: 65924217.4.0000.5248). The legal representative (mother) of the involved patient provided written consent and permission for the publication of data and images.

The delivery and specialized care of the patient were performed in Plantadores de Cana Hospital, in Campos dos Goytacazes, Brazil. The placenta and fetus were immediately collected and fixed accordingly to the techniques described below. Consent and permission were obtained from the patient and the institution for comparison purposes (controls) the following samples were considered: placental tissue obtained from a non-Zika case; and a fetus originated from spontaneous abortion at the same stage of development found in the Zika case. The fetus did not present any other infectious disease.

Histopathological analysis

Samples from the placentae and fetal organs were fixed in formalin (10%), dehydrated in ethanol, clarified in xylene and blocked in paraffin resin. Tissue sections were cut (4 µm thick), deparaffinized in three baths of xylene and rehydrated with decreasing concentrations of ethanol (100 % to 70 %). Sections were stained with hematoxylin and eosin for histological examination. Stained specimens were visualized by light microscopy (Olympus BX 53F, Japan) and digital images obtained by Image Pro Plus software (Version 4.5). All analyses were performed without prior knowledge of the nature of the samples (blind test).

Immunohistochemiscal procedure

For immunohistochemical studies, the paraffin-embedded tissues were cut (4 µm thick), deparaffinized in xylene and rehydrated with alcohol. Antigen retrieval was performed by heating the tissue in the presence of citrate buffer. Next, tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in Tris-HCl (pH 7.4). To reduce non-specific binding, sections were incubated in Protein Blocker solution (Spring Bioscience, USA) for 5 min at room temperature. Samples were then incubated overnight at 4 ºC with anti-human monoclonal antibodies that recognize flavivirus E protein (4G2 - produced in house) Zika NS1 (Arigo, USA), CD8 (DAKOCytomation, USA), CD68 (Biocare Medical, USA), RANTES/CCL5 (Santa Cruz Biotechnology, USA), TNF-α (Abbiotec, USA), IFNγ (Abbiotec), VEGFR2 (Spring Bioscience, USA), all diluted 1:200. In the next day, sections were incubated with a rabbit anti-mouse IgG-HRP conjugate (Spring Bioscience) for 40 min at room temperature. For negative controls, samples were incubated with both antibodies or only with the secondary HRP conjugated antibody. Reactions were revealed with diaminobenzidine (Dako, USA) as chromogen and the sections were counterstained in Meyer's hematoxylin (Dako).

Quantification of positive cells by immunohistochemistry

Slides were evaluated using an Olympus BX 53F microscope. For each specific antibody, 50 images (fields) were randomly acquired at 1000x magnification using the software Image Pro version 4.5 from placentae (Zika infected and control). After collecting

the frames, positive cells were quantified in each of the 50 fields in every organ and the median of positive cell number was determined. All analyzes were accomplished in a blind test without prior knowledge of the studied groups. After quantification, frames exhibited in figures were selected as to be more informative.

Immunofluorescence assay and co-staining of NS1 protein/ phenotypic cell markers:

The paraffin-embedded tissues were cut (4 μm thick), deparaffinized in xylene and rehydrated with decreasing alcohol series. Antigen retrieval was performed by heating the tissue in presence of citrate buffer. In sequence, tissues were blocked with 1% bovine serum albumin (BSA) for 30 minutes and permeabilized with 0.5% Triton X-100 at room temperature. Samples were incubated overnight at 4 ºC with anti-human monoclonal antibodies that recognize Zika NS1 protein (Arigo, USA) and anti-CD11b (Abcam, UK) diluted 1:200 for co-localization. In the next day, sections were incubated with Alexa 488 rabbit anti-mouse IgG (Thermo Scientific) to bind anti-NS1 antibodies and Alexa 555 mouse anti-rabbit IgG (Thermo Scientific, USA) to bind anti-CD11b antibodies. Slides were analyzed under a confocal microscope (Zeiss LSM 510 Meta, Germany).

Statistical analyses

Data were analyzed with GraphPad prism software v 6.0 (La Jolla, USA) using Mann-Whitney non-parametric statistical tests. Significant differences between groups were determined considering $***p < 0.001$.

Table S1. Analysis of the cerebrospinal fluid obtained by lumbar puncture and exams of the patient's peripheral blood.

Liquor			
Test	**Results**		**Reference value**
Cytology	Global: 10 cel/mm^3		-
	Specific: 10% PMN/ 90% MNC/ 0% EOS		-
Total proteins	39 mg/dL		10 - 45 mg/dL
LDH	148 U/L		71- 207 U/L
Glucose	50 mg/dL		50 - 80 mg/dL
Peripheral Blood			
	29/06/2016	**08/07/2016**	
Erythrocytes	2.52	3.51	4.0 – 5.2 millions/ mm^3
Hemoglobin	8.3	11.2	12 - 16 g/dL
Hematocrit	24,1	32,4	35 - 47%
Leucocites	11,000	5,500	4,000 – 11,000/mm^3
Platelets	365,000	336,000	150.000 – 400,000/ mm^3
Erythrocyte sedimentation rate	60	37	0 - 20 mm/h
C-reactive protein	-	2.05	< 0.80 mg/dL
Potassium	3.6	4.2	3.5 - 5.0 mEql/L
Sodium	142	141	135 - 150 mEq/L
Urea	23	14	15 - 45 mg/dL
Creatinin	0.50	0.67	0.4 - 1.4 mg/dL
Aspartate aminotransferase	-	32	10 - 37 U/L
Alanine aminotransferase	-	13	10 - 37 U/L
Total proteins	6.9	9.50	6.0 – 8.0 g/dL
Albumin	4.0	3.31	3.5 – 5.5 g/dL

Table S2. Evaluation of the degree of upper and lower limbs muscle strength (upper and lower limbs, respectively) during period of hospitalization and follow up after hospitalization.

Time of disease evolution (progression)	**Force quantification**				**Functional Severity Scale Hughes Clinic**
	Upper limbs muscle		Lower limbs muscle		
	Proximal	Distal	Proximal	Distal	
14 days	III	II	II	0	04
21 days	IV	II	III	0	05
35 days	IV	III	III	0	04
46 days	IV	III	III	0	04
75 days	V	III	IV	II	03
123 days	V	IV	V	II	03
188 days	V	V	V	IV	02
417 days	V	V	V	IV	02

2.3 **Zika induces inflammation in placenta and BDNF expression can modules fetal microcephaly (Artigo Aceito)**

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Zika induces inflammation in placenta and BDNF expression can modules fetal microcephaly

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Abstract

In Brazil, an epidemic of Zika virus (ZIKV) infections was declared in 2015 that coincided with alarming reports of microcephaly in newborns associated with mother infection. Although the virus has placental tropism, changes in the tissue morphology and immunity of infected patients have not yet been elucidated. Here, we investigated the histopathological and ultrastructural changes along with the immunological profile and the BDNF expression in rare placental material. Tissues were obtained in the 2015-2016 Brazilian epidemic, of ten ZIKV-infected patients during pregnancy, five resulting in cases of fetal microcephaly and five non-microcephaly, compared to five non-infected control placentae. Viral antigens were only detected in samples from the ZIKV infected patients. Infected placentae presented histopathological severe damage, while the ultrastructural evaluation showed abnormal organelles, such as clusters of virus-like particles consistent with the ZIKV dimensions. Increased infiltration of $CD68^+$ and $TCD8^+$ cells, expression of MMPs, cytokines (IFN-γ and TNF-α) and other immunological mediators (RANTES/CCL5 and VEGFR-2) confirmed excessive inflammation and vascular permeability dysfunction. An evaluation of BDNF showed a decrease that could modulate neuronal damage in the developing fetus. The placental changes caused by ZIKV are not pathognomonic, however, the data provide evidence that this infection leads to severe placental injury.

Introduction

Zika virus fever has emerged as an important arbovirus disease whose transmission has impacted numerous regions worldwide. Its etiological agent, Zika virus (ZIKV), was first isolated more than seventy years ago in the Zika forest of Uganda from the blood of sentinel Rhesus monkeys during a 1947 study on yellow fever transmission. For nearly sixty years, serological evidence of ZIKV infections in humans was only detected sporadically on the continents of Africa and Asia. This rare occurance changed in 2007 when an epidemic appeared with a large incidence of the disease in Micronesia that was followed by another in Polynesia in 2013 before its subsequent appearance worldwide (1). In Brazil, a Zika epidemic was declared in 2015 from its appearance in the Northeast of the country that rapidly propagated across the country (1, 2). This epidemic was soon followed by alarming reports of microcephaly in fetuses and newborns that were associated with mothers infected by ZIKV, which led to a declaration by the World Health Organization (WHO) of a public health emergency of international concern (3–5).

Since then, many authors have reported on the capacity of ZIKV to infect neurons and other neuronal cells that most likely detrimentally affect their function and contribute to congenital Zika syndrome (CZS), which has as its main characteristics microcephaly and ventriculomegaly (1, 3–7). In this scenario, studies aiming to understand the mother-fetus interface of ZIKV vertical transmission have been strongly recommended (8). In the vertical transmission, one major barrier is the placenta, a highly specialized organ that ensures the fetus´development, by allowing the exchange of nutrients, solutes and acting as physiological barrier against toxic molecules and pathogens, such as viruses (8, 9). Estimates are that the viral genome can be detected in the placenta of 20%–50% of pregnant women exposed to ZIKV(10). However, the mechanism by which ZIKV crosses the placenta to establish an infection in a fetus has not been completely elucidated.

To date, ZIKV has been identified in amniotic fluid and a range of placental cells (syncytiotrophoblasts, cytotrophoblasts, decidual and endothelial cells) as well as cells of the maternal immune system present in the placenta, such as macrophages and dendritic cells (4, 11–14). At present, definitive evidence is lacking for the histopathological changes associated with a ZIKV infection during an active immune response in the placenta of pregnant patients. Defining these changes could have major implications in

understanding the impact of a positive ZIKV diagnosis for a pregnant mother on the severity of the condition for their fetus as a predictor for microcephaly.

Here, we present the clinical aspects of 10 pregnant patients infected with ZIKV during the outbreak that occurred in Rio de Janeiro between 2015 and 2016. Five of these pregnancies ended with the birth of infants that presented with microcephaly ($ZIKV^{+}MIC^{+}$) and the other five with infant that did not present with microcephaly ($ZIKV^{+}MIC^{-}$). Microcephaly was the only clinical aspect of the newborn considered as it is detectable at the time of delivery and is one of the most prominant characteristics of CZS. Here, we describe the histopathological features observed in both groups of infected placentae with a comparison to five, uninfected control placentae. In addition, we report on the detection of viral antigens in placental cells, the whole profile of immune cells, cytokines, proinflammatory mediators, ultrastructural changes and the detection of virus-like particles by electron microscopy. Finally, we evaluated the expression of brain derived neurotrophic factor (BDNF), an essential factor for fetal brain development, which may be one of the determinant proteins that contribute to the severity of microcephaly due to vertical transmission in ZIKV infection.

Results

Clinical data of pregnant women ($ZIKV^{+}$) with babies that did not present microcephaly (MIC^{-})

Case 1: A 42 year-old patient that reported exanthema and pruritus in the first trimester of gestation. Her serology for IgG against cytomegalovirus, rubella, dengue, toxoplasmosis and HIV were negative. At 39 weeks of gestation, she delivered a baby boy by cesarean that presented a cephalic circumference of 35 cm. The placenta weighed 640 g.

Case 2: A 23 year old patient that reported fever, arthralgia, exanthema and pruritus in the third trimester of gestation. Her IgM serology was positive for Zika, the $PRNT_{90\%}$ was positive (Supplementary Table 1) and the qPCR was positive for Zika in serum (820 copies/ml) and urine (160 copies/ml). Her IgG serology was positive for cytomegalovirus and rubella. The test for dengue NS1 was negative. At 38 weeks of gestation, her baby girl was born by cesarean delivery that presented with cephalic circumference of 37 cm. The placenta weighed 555g.

Case 3: A 21 year old patient that reported fever in the second trimester of pregnancy. Her IgM serology for ZIKV was positive and non-reactive for dengue, chikungunya, rubella, toxoplasmosis, HIV, syphilis and cytomegalovirus. The $PRNT_{90\%}$ was positive for neutralizing antibodies. Her IgG serology was positive for cytomegalovirus and rubella. At 41 weeks of gestation, her baby girl was born by cesarean delivery that presented a cephalic circumference of 36 cm.

Case 4: A 26 year old patient that reported exanthema and arthralgia in the second trimester of gestation. The qPCR was positive for Zika in serum (690 copies/ml) and the $PRNT_{90\%}$ was positive for neutralizing antibodies. At 37 weeks of gestation, her baby boy was born by cesarean delivery that presented a cephalic circumference of 36.5 cm.

Case 5: A 34 year old patient that reported exanthema and pruritus in the third trimester of gestation. At 38 weeks of gestation, her baby girl was born by cesarean that presented a cephalic circumference of 34 cm.

Clinical data of pregnant women ($ZIKV^+$) with babies that presented microcephaly (MIC^+)

Case 6: A 29 year old patient that reported exanthema and pruritus in the second trimester of gestation. Her IgM serology was positive for Zika and the $PRNT_{90\%}$ was positive for neutralizing antibodies. At 38 weeks of gestation, her baby girl was born by cesarean that presented a cephalic circumference of 30 cm.

Case 7: A 24 year old patient that reported exanthema and pruritus in the second trimester of gestation. Her IgM serology for dengue, herpes, chikungunya, rubella, toxoplasmosis, HIV and cytomegalovirus were non-reactive. Her IgG serology was positive for dengue, herpes, rubella and cytomegalovirus, and negative for toxoplasmosis. A dengue NS1 test was negative. At 38 weeks of gestation, her baby boy was born by cesarean that presented a cephalic circumference of 29 cm. The newborn also had ventriculomegaly.

Case 8: A 35 year old patient that reported exanthema, shiver and pruritus in the first trimester of gestation. Her IgM serology was positive for Zika and the $PRNT_{90\%}$ was negative for neutralizing antibodies. Her IgG serology for cytomegalovirus and rubella were positive while negative for dengue, toxoplasmosis and HIV negative. At 38 weeks of gestation, her baby girl was born by cesarean with a cephalic circumference of 29 cm.

Case 9: A 25 year old patient that reported exanthema and pruritus in the third trimester of gestation. Her IgM serology was positive for Zika and the $PRNT_{90\%}$ was positive for neutralizing antibodies. At 37 weeks of gestation, her baby girl was born by cesarean with a cephalic circumference of 27 cm. The placenta weighed 565g.

Case 10: A 28 year old patient who experienced fever in the third trimester of pregnancy. Her IgM serology for dengue, chikungunya, rubella, toxoplasmosis, HIV, syphilis and cytomegalovirus were non-reactive, and positive for ZIKV. Her IgG serology was positive for cytomegalovirus and rubella. At 38 weeks of gestation, her baby boy was born by cesarean delivery with a cephalic circumference of 28 cm. The baby also presented artrogriposis, with lower and upper limb involvement. The placenta weighed 670g.

Histopathological analysis

The histopathological analysis of control samples showed a regular arrangement of the decidual layer and normal chorionic villi that included syncytiotrophoblasts, cytotrophoblasts and endothelial cells (**Figures 1A–C**), which suggested that the collection and fixation of placental samples was adequate. For the evaluation of the placenta samples from ZIKV mothers, the full range of samples were imaged and qualified for histological alterations. In infected placentae, we observed relevant damage in the decidua and chorionic villi (**Figures 1D–R**). Large areas of immature chorionic villi were evident that included inflammatory changes seen as acute deciduitis and villositis, chronic villositis (lymphocytic infiltrate), fibrous endothelial thickening, vascular and intervillous congestion and focus of intevillositis. Other alterations were present: calcification, edema, fibrin deposits and villous hypoplasia. In addition, a few incidences of ischemic lesions were identified as infarct and decidual vasculopathy (fibrinoid necrosis) (Supplementary Table 1). No correlations were apparent with the presentation of microencephaly in infants after birth suggesting that the changes observed represented the effects that can be expected in placenta from a maternal ZIKV infection that are not predictive for the impact on fetus development.

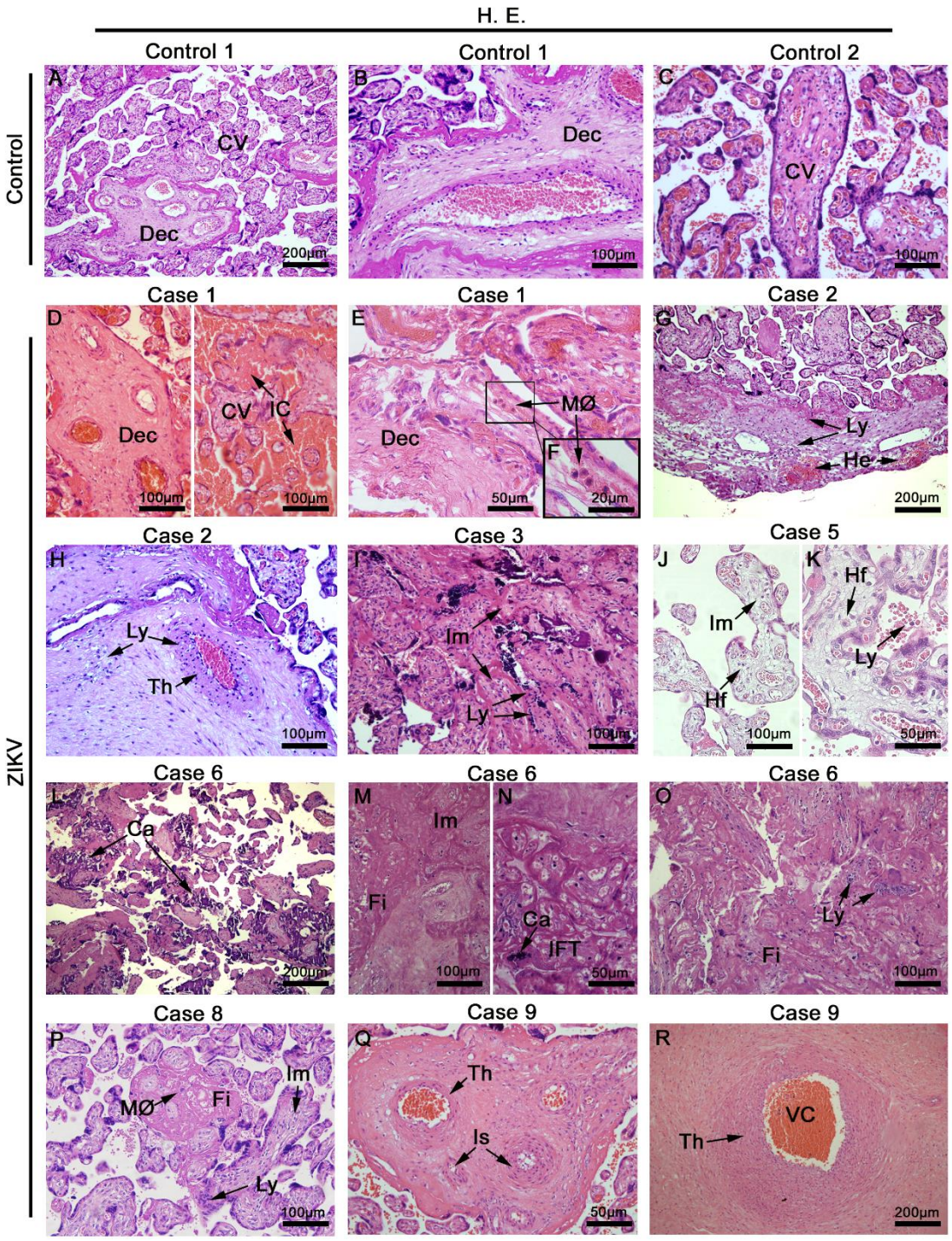

Figure 1- Placentae histopathology. A-C) Placentae from non-ZIKV patients stained with H&E and presenting normal features: maternal decidua (Dec) and chorionic villi (CV). **(D-R)** Placentae from ZIKV infected patients that presented a range of different alterations such as vascular congestion (VC), intervillous congestion (IC), macrophage infiltrate (MØ), lymphocytic infiltrate villous (Ly), hemorrhage (He), endothelial thickening (Th), immature chorionic villi (Im), Hofbauer cells (Hf), calcification (Ca), fibrin areas (Fi), infarct (IFT) and ischemia (Is).

Ultrastructural alterations and Zika virus particles

No evidence of ultrastructural changes was observed in placental cells from control patients, represented in images form a single control 1 (**Figures 2A-D**), which again suggests the the collection and treatment of samples maintained their structural integrity. The cytotrophoblasts presented normal aspects for all organelles, including the mitochondria and the nucleus, which was heterochromatic (**Figure 2A-B**). Syncytiotrophoblasts presented an electron dense cytoplasm, as expected, with heterochromatic nuclei (**Figure 2C**). In the extracellular matrix, collagen filament structures were readily identified in transverse and longitudinal sections (**Figure 2D**). In contrast, the analysis of infected placentae showed cytotrophoblasts with little nuclear variation and an electron lucid cytoplasm containing swollen mitochondria showing a loss of cristae and ruptured membranes (**Figure 2E-F**). The syncytiotrophoblasts aspects were extensively modified with an enlargement of vesicles and apoptotic bodies along with an absence of their normal membrane extensions and secretions of microvesicles (**Figures 2E and G**). An investigation of the extracellular matrix across the images of placenta samples from the different donors did not reveal collagen filaments, which suggests a decrease in this matrix component (**Figure 2H**). Multiple occurrences of clusters were identified that presented a profile reminescent of virus-like particles, which were often positioned adjacent to the endoplasmic reticulum of cytotrophoblast cells. These particles were measured to have a diameter of ~25 nm in diameter, which is consistent with the dimensions of ZIKV (**Figure 2I-K**).

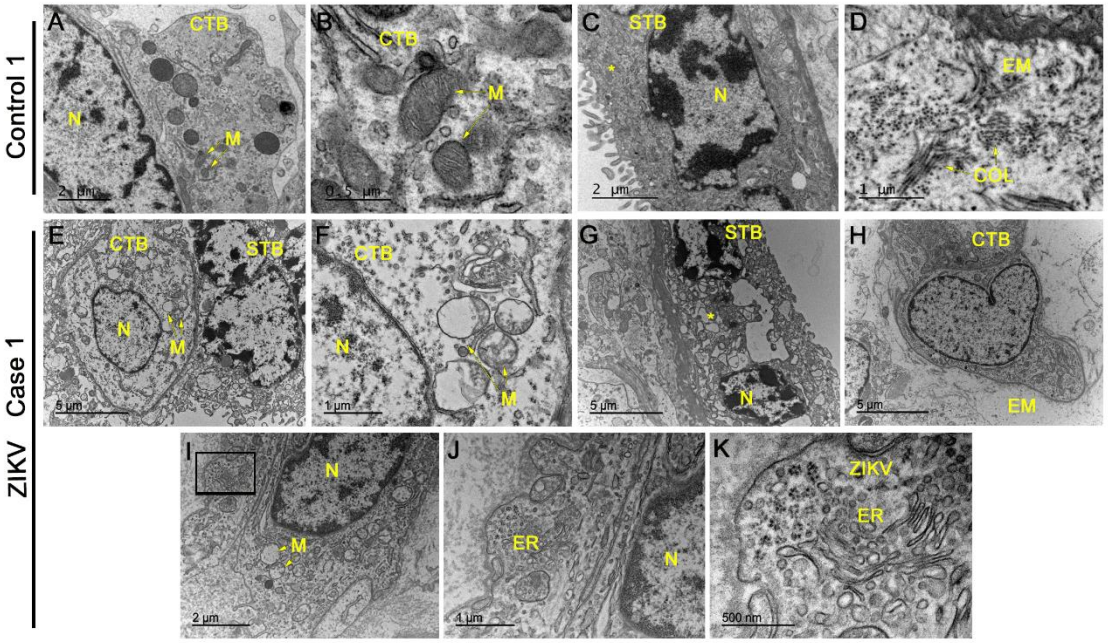

Figure 2- Electron microscopy analysis of placental sections showed alterations and virus-like particles in ZIKV infected samples. A–D) Electron microscopy images of ultrathin sections of placental tissue from a single, non-ZIKV infected mother that exhibited regular cytotrophoblasts (CTB), syncytiotrophoblasts (STB), nucleus (N), mitochondria (M) and collagen filament structures (COL). **E-H)** Electron micrographs of ultrathin sections of placental tissue from different ZIKV-infected mothers showing CTB with alterations in the cytoplasm, nuclear variation (N) and swollen mitochondria with a loss of cristae and membrane rupture. The identified STB presented an enlargement of vesicles and apoptotic bodies (asterisks) along with an absence of normal membrane extensions and evidence of microvesicle secretion. The extracellular matrix (EM) did not present collagen filaments. **I-K)** Identification of clusters of virus-like particles that were positioned near the endoplasmic reticulum (ER) of CTB and ~25 nm in diameter, which is consistent with the dimensions of ZIKV.

Viral detection in the placentae

The placental tissue samples were screened for the presence of ZIKV E and NS1 protein using immunohistochemistry. These viral antigens were detected in all samples obtained from infected patients, while immunostaining was negative in samples of control placentae (**Figures 3A and E**). The E structural protein was detected in decidual cells and in syncytiotrophoblasts as well as endothelial and mesenchymal cells of chorionic villi (**Figures 3B-D**). The NS1 protein was also detected in cytotrophoblasts, syncytiotrophoblasts and mesenchymal cells, moreover in Hofbauer cells of chorionic villi and in decidual cells (**Figures 3F-H**). Viral antigens were detected mainly within the cytoplasmic region of cells with minor to indefinite staining in the nuclear area. The anti-NS1 antibody used in these assays is ZIKV specific, therefore, it was able to differentiate ZIKV from other flaviviruses. Additionally, the replication was also confirmed by *in situ* hybridization using a probe that anneals only to the negative strand of the ZIKV RNA, which revealed the presence of this RNA in decidual cells, syncytiotrophoblasts, cytotrophoblasts and villous mesenchymal cells (**Figures 3J-L**). All controls were negative for the immunohistochemistry and *in situ* hybridization (**Figures 3I**). The staining pattern observed strongly suggests that the replication of ZIKV was in progress at the time of birth.

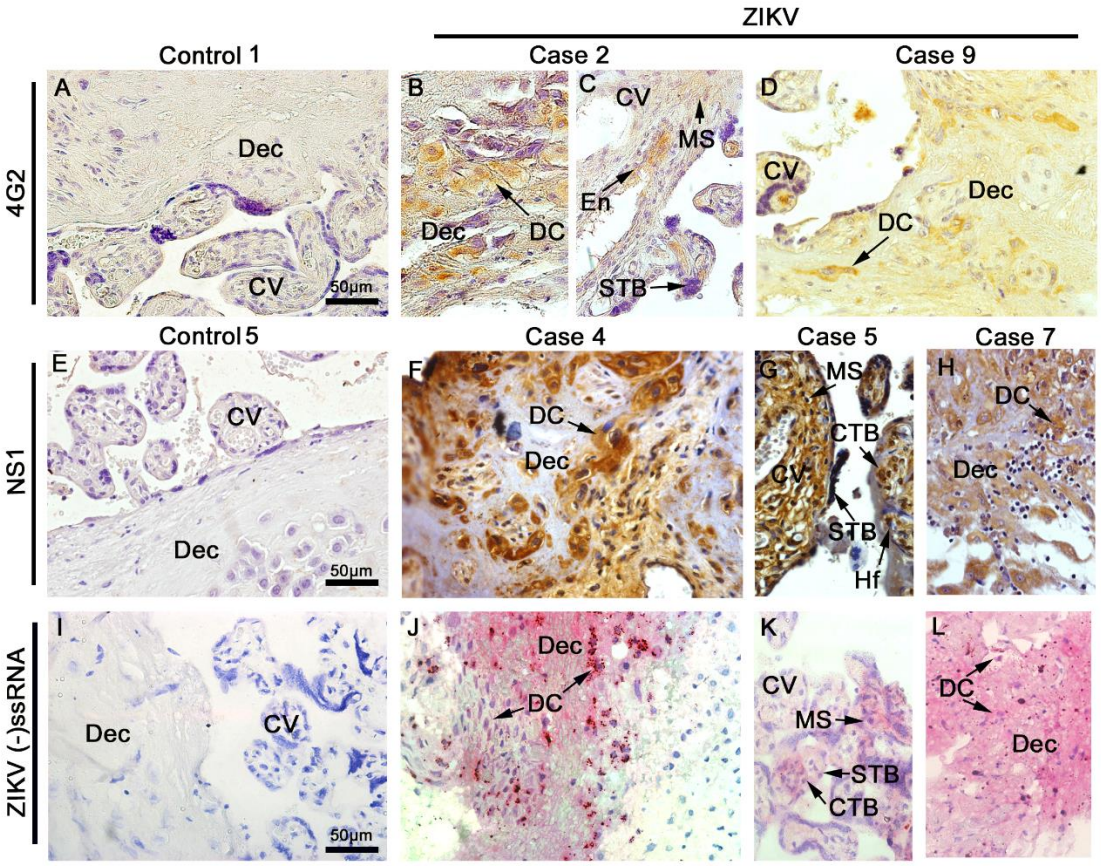

Figure 3- Detection of viral antigens. Tissue sections were probed by immunohistochemistry for the E (**A-D**) and NS1 (**E-H**) antigens of ZIKV as well as by *in situ* hybridization for the genome of ZIKV (**I-L**). Representative images of control placentae (**A, E and I**) showing the absence of reactivity for the antigens and genome of ZIKV. Representative images of placenta from ZIKV^{+} placenta, independent of the cephalic circumference of the infant, show: (**B-D**) the presence of E protein in decidual cells (DC) of decidua (Dec), mesenchymal cells (MS), endothelial cells (En) and syncytiotrophoblast (STB) of chorionic villi (CV) that was independent of the cephalic circumference of the infant; (**F-H**) NS1 protein detected in decidual cells (DC) of decidua (Dec), mesenchymal cells (MS), Hofbauer (Hf), syncytiotrophoblast (STB) and cytotrophoblast (CTB) of chorionic villi (CV); and (**J-L)** Detection of ZIKV RNA negative strand by *in situ* hybridization in decidual cells (DC) of decidua (Dec), mesenchymal cells (MS), syncytiotrophoblast (STB) and cytotrophoblast (CTB) of chorionic villi (CV).

Characterization of Cell Subpopulations

To gain further insight into the subpopulations of immune cells that could be migrating to inflamed tissues, immunohistochemistry was performed to characterize the cell types present in the placentae. A significant upsurge in the number of $CD68^+$ cells were detected in both groups of infected placentae ($ZIKV^+MIC^-$ and $ZIKV^+MIC^+$), which suggested a recruitment of macrophages and hyperplasia caused by the infection in the basal decidua and Hofbauer cells (**Figures 4C-F**). The increase of macrophages were quantified to be 5- and 6-fold in placentae of $ZIKV^+MIC^-$ and $ZIKV^+MIC^+$ groups, respectively (**Figure 4G**). Even though T $CD8^+$ cells were found in the same areas (**Figures 4J-M**), few $CD4^+$ cells were detected within the tissues (Supplementary Fig. 1). The T $CD8^+$ lymphocytes were increased 7- and 8-fold in the tissues from $ZIKV^+MIC^-$ and $ZIKV^+MIC^+$ groups, respectively (**Figures 4N**). The control placentae showed a low number of positive cells for both markers (**Figures 4A-B and H-I**). Additional evidence for the replication of ZIKV in macrophages was observed by the colocalization of NS1 protein with the CD163 marker for differentiated macrophages in dual stained immunofluorescent images (**Figure 4P**). As expected, no signals were observed for NS1 in control tissue (**Figure 4O).**

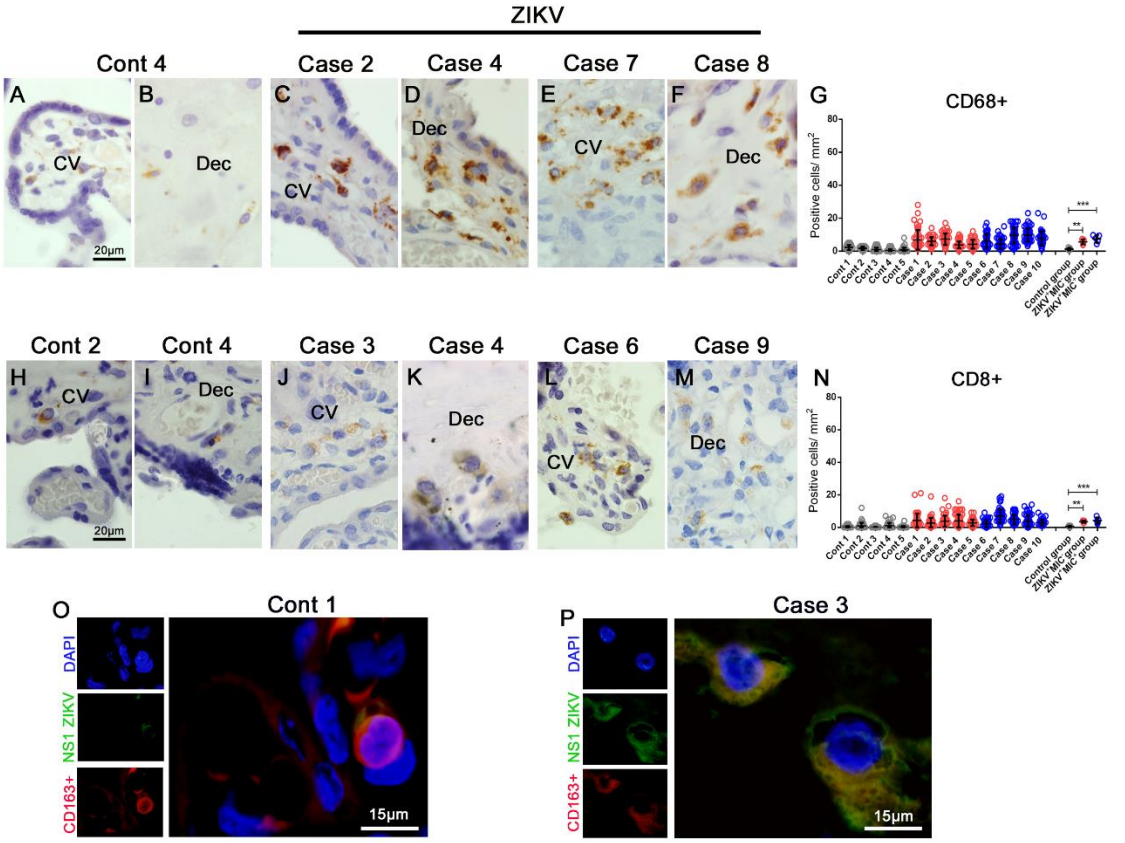

Figure 4- Increased cellularity of mononuclear cell subpopulations in ZIKV-infected placental tissues. CD68 and CD8 were detected in placenta samples by immunohistochemistry. **A-B)** $CD68^+$ cells in decidua and chorionic villi of control placenta. **C-F)** $CD68^+$ cells in decidua and chorionic villi of ZIKV infected placentae. **G)** Quantification of $CD68^+$ cells showing a 5 or 6-fold increase in placentae of $ZIKV^+MIC^-$ and $ZIKV^+MIC^+$ groups, respectively. **H-I)** $CD8^+$ cells in decidua and chorionic villi of control placentae. **J-M)** $CD8^+$ cells in decidua and chorionic villi of ZIKV infected placentae. **N)** Quantification of $CD8^+$ cells showing a 7 or 8-fold increase in the tissues of $ZIKV^+MIC^-$ and $ZIKV^+MIC^+$ groups, respectively. **O-P)** Immunofluorescence for the presence of ZIKV NS1 protein (green) and CD163 (red; biomarker for macrophages) showing colocalization. Nuclei were stained using DAPI (blue). **O)** ZIKV NS1 antigen was not detected in the control placenta.

Cytokines and Mediators profile in the placentae

Based on the inflammatory infiltrate observed in H&E stained sections and the detection of an increase in number of immune cells in infected placentae, the production of cytokines and mediators were investigated. The expression of TNF-α and IFN-ɣ was evaluated due to their participation in a pro-inflammatory response. In addition, Additionally, the markers VEGFR-2 and RANTES/CCL5 were included as they have been implicated with an alteration in vascular permeability. In control samples, all markers were detected at low levels (**Figures 5A-H**). TNF-α expression was diffuse in Hofbauer and mesenchymal cells in the chorionic villi and in decidual cells (**Figure 5A**). Its expression was 12-fold higher in the $ZIKV^{+}MIC^{-}$ group and 16-fold higher in the placenta from the $ZIKV^{+}MIC^{+}$ group (**Figure 5B**). IFN-ɣ was found mostly in decidual cells and decidual macrophages (**Figure 5C**), with a 3- and 5-fold increase in the $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 5D**). The expression of VEGFR-2 was found in endothelial and mesenchymal cells in chorionic villi as well as in circulating macrophages within the vessels and in endothelial cells in decidua (**Figure 5E**). This receptor had an increased expression level of 11- and 13-fold in the $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 5F**). The chemokine RANTES/CCL5 was detected mainly in the endothelium and in Hofbauer cells located within the chorionic villi and in decidual cells and syncytiotrophoblasts of the decidua (**Figure 5G**). RANTES/CCL5 was showed 4- and 5-fold greater expression in $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 5H**). The statistical analysis of the expression of all these markers determined that they were significantly increased in the $ZIKV^{+}$ patient placentae compared to $ZIKV^{-}$ control tissues. Their expression was even higher in the placentae of patients who had babies with microcephaly, with a greater relevance in statistical significance. (**Figures 5B, D, F and H**).

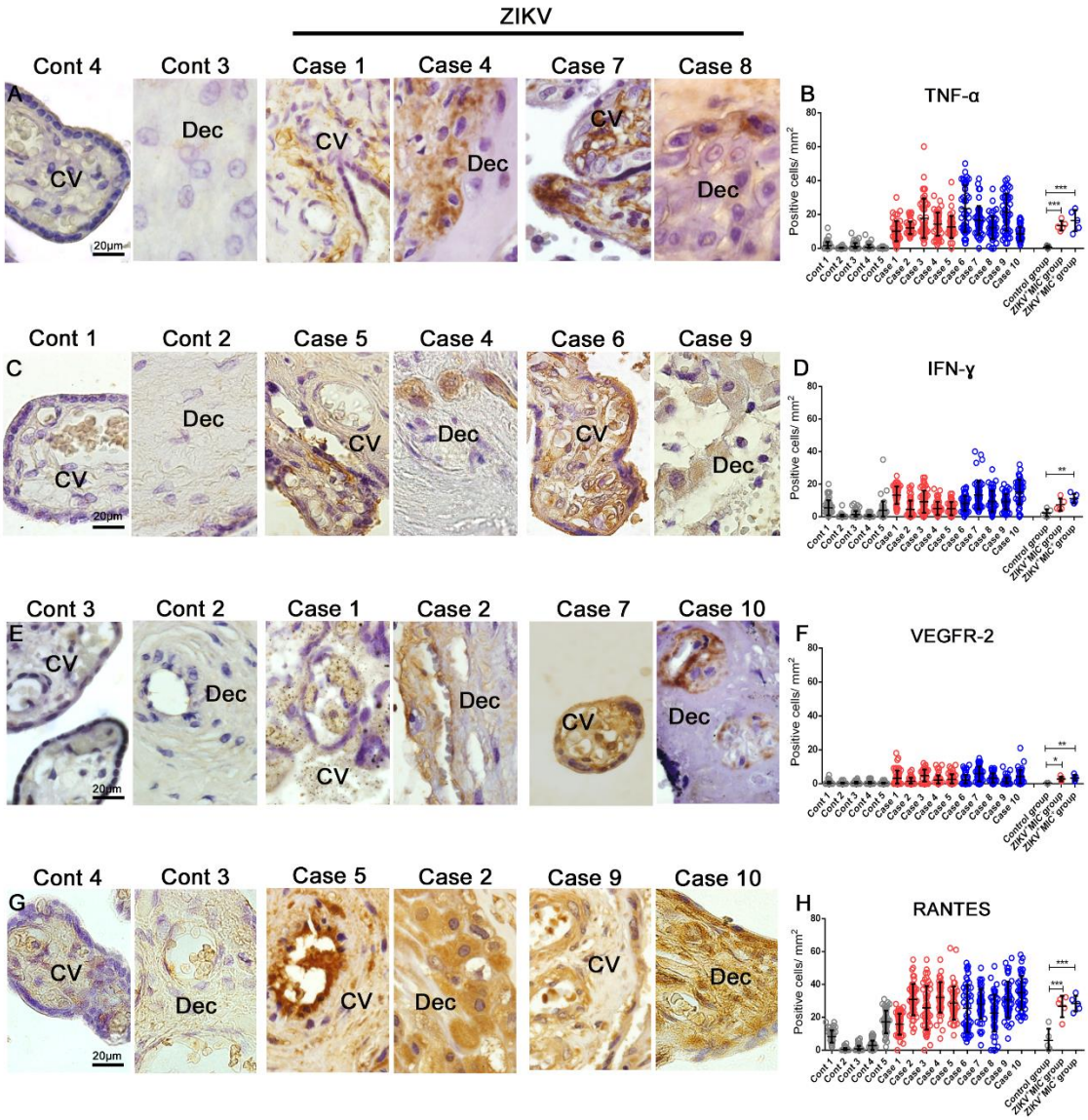

Figure 5- Cytokine-producing cell profile. Detection of TNF-α, IFN-γ, VEGFR-2 and RANTES/CCL5 by immunohistochemistry show **A)** TNF-α in cells of chorionic villi in control placentae (Left panel) and ZIKV infected placentae (Right panel). **C)** Production of IFN-γ in macrophages as well as endothelial cells in chorionic villi and decidual cells of the decidua of control placentae (Left panel) and ZIKV infected placentae (Right panel). **E)** VEGFR-2 was expressed in endothelial cells of decidua and chorionic villi in control placentae (Left panel) and ZIKV infected placentae (Right panel). **G**) RANTES/CCL5 present mainly in the endothelium and Hofbauer cells located within the chorionic villi and decidual cells and syncytiotrophoblasts of the decidua in control placentae (Left panel) and ZIKV infected placentae (Right panel). Quantification of the cells positive for **B), D, F and H)** Quantification of the number of cells expressing TNF-α (**B**), IFN-γ (**D**), VEGFR2 (**F**) and RANTES/CCL5 (**H**) showed an increased expression of local pro-inflammatory cytokines and mediators in ZIKV positive placentae compared to controls.

Changes in placental collagen and matrix metalloproteinases

The absence of collagen in the electron micrographs was confirmed by its specific staining with Picro Sirius Red, which showed that a ZIKV infection led to a drastic decrease in placental collagen (**Figure 6A**). The reduction was 5- and 9-fold in the tissues from $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 6B**). The levels of collagen can be altered by matrix metalloproteinases (MMPs), which can degrade collagen and are known to play a crucial role in pregnancy. MMPs are increased during inflammation from their production by the infiltrated immune cells. An investigation of MMP-2 and MMP-9 levels showed that both proteins were expressed at low levels in decidual cells and chorionic villi cells of control placenta. However, their expression was substantially elevated in the placenta from $ZIKV^{+}$ mothers and displayed a diffuse pattern (**Figures 6C and E**). MMP-2 levels were 6- and 8-fold greater in $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 6D)**. MMP-9 increased 11- and 10-fold in $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups, respectively (**Figure 6F).**

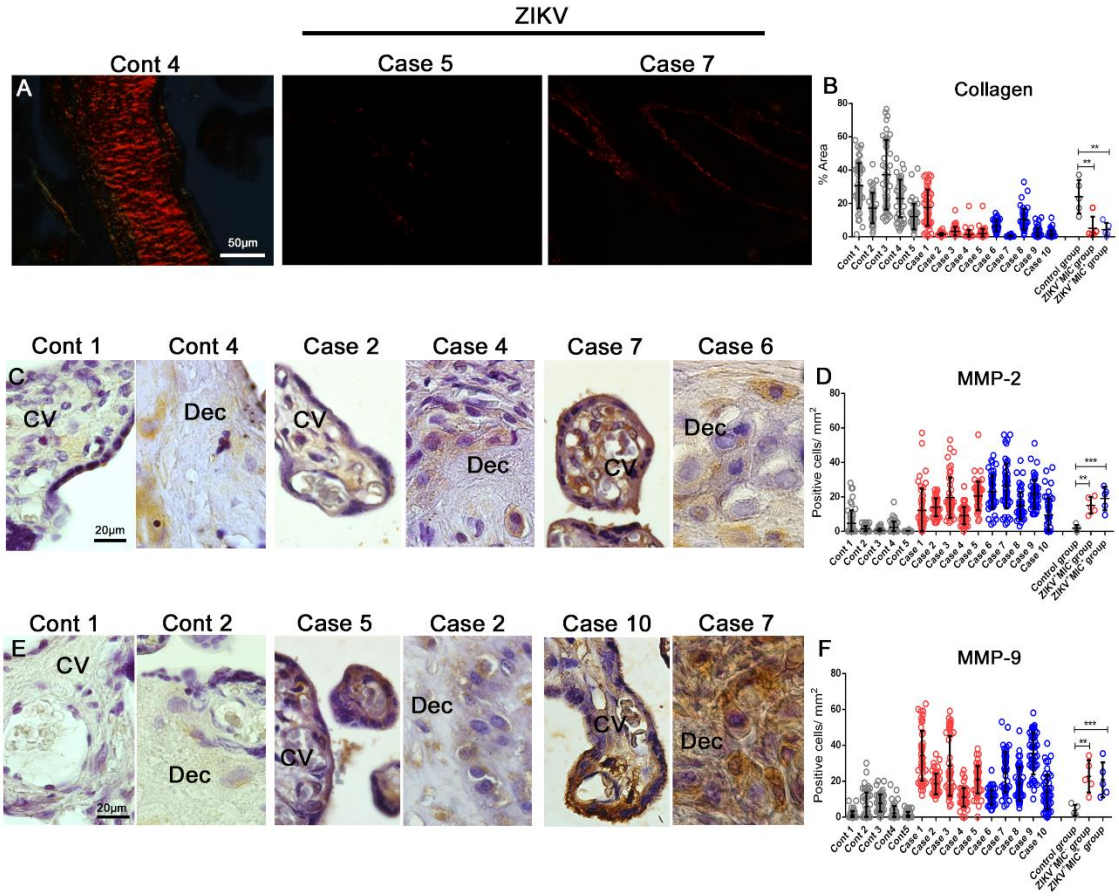

Figure 6- Detection and quantification of collagen, MMP-2 and MMP-9 collagenases expression. A) Collagen detection by Picro Sirius Red staining in placental tissues. **B)** The percent collagen area was quantified in all cases that showed a decrease in the expression of collagen in infected placentae. **C-E)** Detection of MMP-2 and MMP-9 in decidual cells and cells located within the chorionic villi in both control and ZIKV infected placentae. **D-F)** Quantification of the number of cells expressing MMP-2 and MMP-9 showed an increased expression in ZIKV infected placental tissues.

BDNF expression in placental cells

Lastly, the placental expression of an important neurotrophine related to neurogenesis, BDNF, was detected and quantified by immunohistochemistry. In control samples, BDNF was readily detected by an intense and diffuse signal in cells of decidua and chorionic villi (**Figure 7A-B**). The intensity was noticeably diminished in samples from ZIKV^{+} placentae and the number of BDNF expressing cells was considerably lower (**Figure 7C-F**). By quantification, their numbers decreased from 12.35 BDNF positive cells/mm^2 in the control group to 4.25 in the ZIKV^{+}MIC- group, which was not statistically signification. In the ZIKV^{+}MIC^{+} group, only 0.7 BDNF positive cells/mm^2 were detected, which was significantly different (**Figure 7G**).

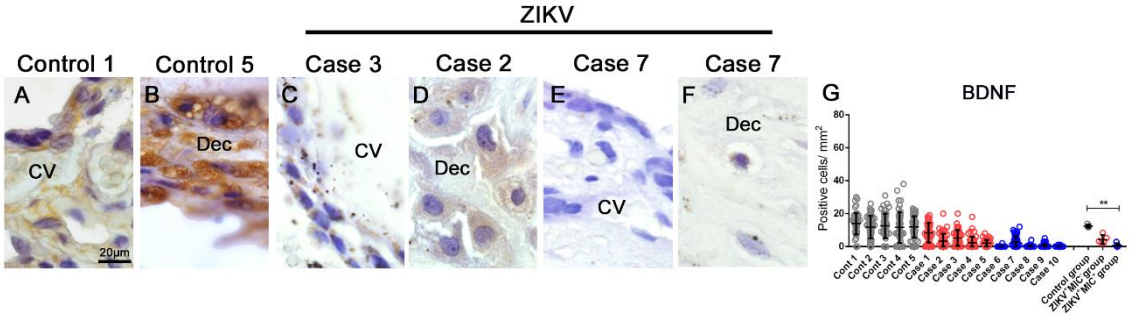

Figure 7- BDNF detection and quantification in placental tissues. A-F) Detection of BDNF in cells of chorionic villi and maternal decidua of controls and ZIKV infected placentae by immunohistochemistry. **G**) A quantification of the number of cells expressing BDNF showed a decreased in the expression of this hormone in ZIKV^{+} MIC^{+} cases.

Discussion

Here, we investigated the impact of a maternal ZIKV infection on placental tissue in patients who gave birth to babies with or without microcephaly during the ZIKV outbreak in Brazil. The histopathology of the ZIKV infection on placenta in Brazilian patients has been studied previously by our group and some alterations are common in most cases, such as a delayed villi maturation, fibrin deposits, calcification and inflammatory changes in villi and the decidual layer (4, 14). The main alteration observed in the placentae of the cases studies was the delay in villi maturation, confirmed by other groups (15–17). These histopathological alterations are similar to those described in the placentae from $ZIKV^{+}$ women in French Guiana, such as villitis, intervillositis, calcification, infarct, ischemia, inflammatory infiltrate and fibrin deposits (18–20). Overall, the placental changes discovered in ZIKV infection are non-pathognomonic and often have particular characteristics in different patients.

In addition to confirming the histopathological modifications associated with a ZIKV infection, we examined cells of the placentae at an ultrastructural level. The intracellular damage caused by the virus was observed principally in mitochondria of cytotrophoblasts. There also was an increase in the vesicles present in the syncytiotrophoblasts and it was difficult to detect collagen filaments in the samples from infected patients. While different ultrastructural damage has been observed previously with ZIKV infections, this is the first report on modifications in the structure of organelles in placental tissue obtained from a human *in vivo* infection (4, 21). In addition to the organellar alterations, the presence of clusters of virus-like particles were identified positioned near to the endoplasmic reticulum of cytotrophoblasts.

The viral antigens E and NS1 as well as the genomic RNA of ZIKV were detected in numerous cells throughout the placentae, in both fetal or maternal portions that included trophoblasts. These results provide further proof that placental cells are susceptible and permissive to ZIKV infection, which is consistent with the hypothesis that ZIKV can reach the developing fetus by progressive cell to cell infections that can penetrate the placental barrier (4, 14, 19, 22, 23). It is important to note that results presented here provides ample evidence that the ZIKV established a persistent, replicating infection in the placenta months after the reported onset of the acute infection based on the detection of virus by RT-PCR, electron microscopy and immunohistochemistry as

well as markers of replication from *in situ* hybridization and viral antigens at the moment of delivery.

Hofbauer cells and decidual macrophages are residents in the placenta that have a regulatory role in pregnancy to maintain a homeostatic environment, which is essential for fetal development (24). We detected a large increase in the number of macrophages in placenta from infected mothers. In addition, fluorescence microscopy captured colocalization of ZIKV NS1 in $CD163^+$ activated macrophages that suggested these cells were sites of virus replication. Macrophages have been previously identified as principal targets for ZIKV infection and could provide a pathway for the vertical transmission of ZIKV through their activation that can lead to a prominent and diffuse hyperplasia (14, 19, 25). Infected $CD163^+$ cells have already been suggested as one of the factors associated with virus delivery to the fetus that lead to ZIKV-induced fetal damage (26).

In the samples analyzed in this study, we found an expressive increase in the numbers of T $CD8^+$ lymphocytes. Regla-Nava and colleagues suggested that the lack of T $CD8^+$ cells, which occurs in mice exhausted by a previous infection, such as dengue fever, could facilitate ZIKV infection (27). Most patients in our study were IgG negative or not reactive for dengue suggesting that none were compromised by a previous infection that have facilitated their ZIKV infection. Even the exception, case 7, still had an increase in the migration of T $CD8^+$ cells to the placenta. This increase in T $CD8^+$ lymphocytes has been observed in non-human primates after the decrease of viremia, which suggests a protective role for T $CD8^+$ cells in controlling ZIKV replication (28). In humans, while few reports have shown the cellular profile in the placenta from ZIKV infected mothers, our observation from a pregnancy of only 15 weeks showed the same characteristics (14). Another case report that presented as positive for T $CD8^+$ lymphocytes was even more expressive for T $CD4^+$ (19), which was unlike our samples where the number T $CD4^+$ cells were insufficient for quantification.

The increase in macrophages and T $CD8^+$ cells characterizes a chronic inflammatory environment in the placenta, with lesions such as deciduitis and villitis observed in all cases (29). Immunity is essential for the development of a pregnancy, from implantation to delivery, and it is now known that maternal immune activation (MIA) is dynamic and normally very effective at preventing viral infections (30–32). However, ZIKV appears to establish a placental infection that bypasses the MIA and promotes inflammation. This environment can be initiated through the release of pro-inflammatory

cytokines, such as TNF-α and IFN-ɣ whose levels are exacerbated in this study, which induce chemotaxis and cellular activation that also increase the expression of MHC-1 for even more intense actions by cytotoxic lymphocytes.

The levels of VEGFR-2 receptor and RANTES/CCL5 mediator were also elevated in the tissues studied, which can lead to an increase in vascular permeability and could cause a large circulatory dysfunction as the fibrin deposits. Fibrin deposits in the placenta can observed in cases of spontaneous abortion, premature birth and fetal death, which suggests a direct affect on the development of the fetus and pregnancy (33). The expression of VEGFR has already been related to other pathologies in the placenta and RANTES/CCL5 has been previously observed in ZIKV placental infection (14, 25, 34, 35). Their changes can lead to edema and a failure in the distribution of nutrients as well as hormones necessary to maintain tissue homeostasis.

In our study, placental tissues infected with ZIKV showed a large decrease in the expression of collagen, which corroborated findings from the ultrastructural analysis and was consistent with the higher production levels of MMP-2 and MMP-9 enzymes. The extracellular matrix (EM) provides an environment conducive to placental development, regulating cellular functions such as signaling, proliferation, migration and invasion. EM is composed of proteoglycans, glycosaminoglycans and has collagen as its main structural component. Most placental collagen is type III (around 60%), followed by type I collagen (approximately 30%) and the other types are IV, V and VI (36, 37). MMPs play a role in the implantation, vasodilatation and separation of fetal membranes, developing a crucial role in collagen degradation according to the signaling by hormones (38, 39). It is known that the inflammatory environment leads to the release of MMPS by immune cells (40). Due to the highly inflammatory environment caused by ZIKV infection, it is certain that the immune cells have secreted and caused this increase in MMPs and consequently the degradation of collagen, leading to malefic tissue remodeling for placental homeostasis. The increase in MMPs in infected placentas may have contributed to the immaturity of the villi.

In all analyses of immune cells and cytokines profile, there were no statistical differences observed between the samples of $ZIKV^{+}MIC^{-}$ and $ZIKV^{+}MIC^{+}$ groups. In some quantifications, there was a difference in the p value (probability of significance) in each of these groups separately compared to control group, with $ZIKV^{+}MIC^{-}$ group always having a lower p-value. The only exception was the IFN-ɣ, where there was no

difference between the $ZIKV^{+}MIC^{-}$ group and the control, even though there was an increase. Our results showed that there is a large inflammation response in the placenta from mothers with a ZIKV infection, but if it has a role in the changes in brain fetal development, it is subtle.

In the absence of a clear role for the inflammation response in the presentation of micrencephaly, the amount of placental BDNF, a factor described as a determinant for fetal brain development, was evaluated (41, 42). BDNF is a neurotrophic factor that is produced in placental tissue and plays an important role in cytotrophoblast differentiation and proliferation (43, 44). Additionally, this neurotrofin promotes neuronal growth and differentiation in the central and peripheral nervous system during fetal development (45, 46). The placentae of patients infected with ZIKV, especially from the group that presented infants with microcephaly, showed a decrease in BDNF expression, which suggests that BDNF levels in the placenta could serve as predictive marker for the extent of damage during fetal brain development.

Conclusion

As summarized in **Figure 8**, our current study uncovered many placental cells that are susceptible and permissive to ZIKV infection. In addition, there is a large involvement of immune cells and pro-inflammatory cytokines in the infected tissue, leading to changes in activation and recruitment of circulating cells as well as alterations in the extracellular matrix and vascular permeability. Statistically, the inflammatory response in the placenta did not have a discrete impact on the presentation of microcephaly, subtle differences were evident and an expanded study may uncover relevant makers. BDNF, which is important in the development of the brain, was found in the placenta could be a promising marker for predicting the impact of a maternal ZIKV infection on fetal brain alterations. As infected, pregnant women are the main target audience for a possible vaccine against Zika, knowledge of the immune cells involved in placental inflammation, including the cytokines and mediators released by local cells, in the course this disease is crucial for its development. The discoveries from this study highlight this need and advance the current description placental change that contribute to congenital ZIKV pathogenesis.

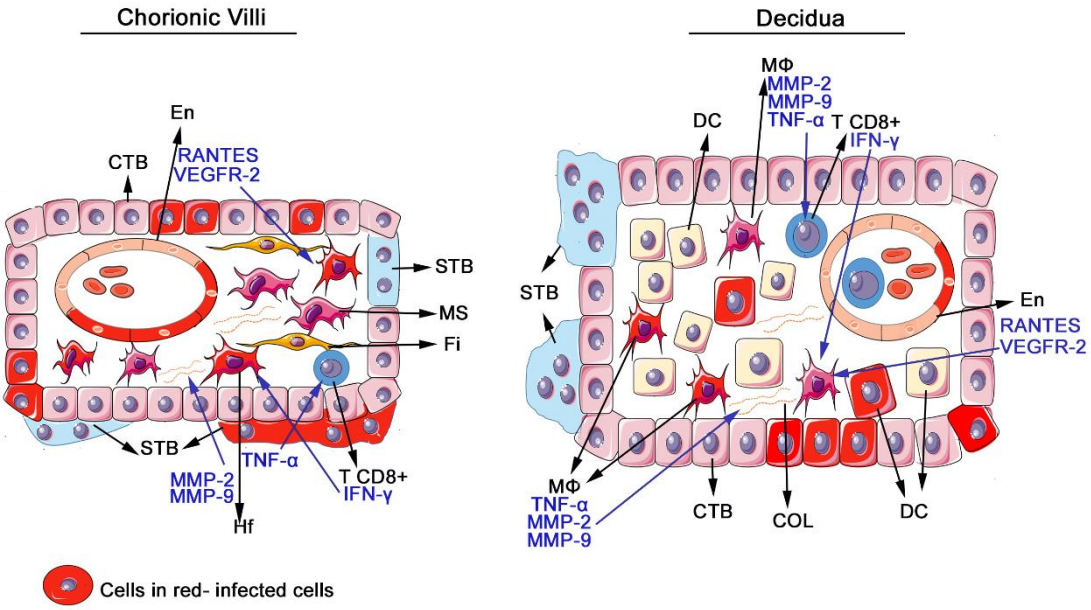

Figure 8- Schematic drawing of placental cells detected infected with ZIKV and cytokines and mediators produced by the mononuclear inflammatory infiltrate. The scheme represents the functional unit of the placenta (chorionic villus) and the maternal region (decidua), comprised of cytotrophoblast (CTB), endothelial cells (En), syncytiotrophoblast (STB), mesenchymal cells (MS), fibroblasts (Fi), Hofbauer (Hf), macrophages (MØ), decidual cells (DC) and extracellular matrix, containing collagen (COL). In the placentae of infected patients, ZIKV antigens were detected in all these cells and there was an increase in the number of macrophages and Hofbauer cells, which are responsible for the production of matrix metalloproteinases that degrade the collagen as well as TNF-α that activates and attracts other immune cells. There was also an increase in T $CD8^+$ lymphocytes, responsible for the production of IFN-ɣ, which further activates macrophages. In endothelial cells, there was large expression of VEGFR-2 and RANTES, which increases vascular permeability and are able to induce macrophages migration.

Material and Methods

Ethical statements and sample collection

All procedures performed during this study were approved by the Ethics Committee of the Oswaldo Cruz Foundation/ FIOCRUZ (CAEE: 65924217.4.0000.5248) and by the Ethics Committee of Faculty of Campos Medicine/Benedito Pereira Nunes Foundation (CAEE: 65924217.4.3001.5244). Consent and permission were obtained from patients and participating institutions. Ten placentae were collected from women infected by ZIKV during pregnancy that resulted in the birth of five babies with birth microcephaly ($ZIKV^{+}MIC^{+}$) and five with normal cranial circumference at birth ($ZIKV^{+}MIC^{-}$). After delivery, placenta samples were fixed in 10% formalin or 2.5% glutaraldehyde. Samples from ZIKV infected women were collected at the Hospital Plantadores de Cana, Hospital Geral Dr. Beda from Campos dos Goytacazes, RJ, Brazil and Hospital de Clínicas Padre Miguel, from Rio de Janeiro, RJ, Brazil. As a reference control, five samples of term placenta from healthy donors were included. All samples were collected between 2015-2016 that coincide with the ZIKV epidemic in Brazil.

Histopathological investigation

Fixed placenta samples were dehydrated in ethanol, clarified in xylene and blocked in paraffin. Tissue sections (4 μm thick) were mounted onto glass sides, deparaffinized in three baths of xylene and rehydrated with decreasing concentrations of ethanol (100 to 70%) before staining with hematoxylin and eosin for histological examination. Prepared specimens were observed by light microscopy (Olympus, Japan) and digital images captured using Image-Pro Plus software version 7. All images were coded to blind evaluators to $ZIKV^{+/-}$ and $MIC^{+/-}$ prior to analysis.

Morphometry

Collagen was revealed by Picro Sirius Red and slides were observed under polarized light microscopy (Olympus). Fifty fields were randomly acquired at 400x magnification from across the placenta samples (Zika-infected and control) and the area

of collagen was measured to calculate the percentage of collagen area (collagen area/total area of the image).

Immunohistochemistry assays

Paraffin-embedded tissue sections (4 μm) were mounted onto glass slides, deparaffinized in xylene and rehydrated with alcohol. Antigen retrieval was performed by heating the tissue in the presence of citrate buffer (pH 6.0). Next, tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in PBS (pH 7.4). Samples were then incubated overnight at 4 ºC with anti-human monoclonal antibodies against: flavivirus E protein (4G2 - produced in house), CD8 (DAKO Cytomation, USA), CD68 (Biocare Medical, USA), CD4 (Cell Marque, USA), RANTES/CCL5 (Santa Cruz Biotechnology, USA), TNF-α (Abbiotec, USA), IFNγ (Abbiotec), VEGFR2 (Spring Bioscience, USA), Zika NS1 (Arigo, USA) or BDNF (Sigma-Aldrich, USA) at suggested dilutions. After three washes, sections were incubated with an anti-mouse or anti-rabbit IgG-HRP conjugate (Spring Bioscience) for 40 min at room temperature. HRP was revealed by its activity on the chromogen substrate diaminobenzidine (Dako, USA) and sections were counterstained in Mayer's hematoxylin (Dako). For negative controls, samples were incubated with either only primary antibodies or secondary HRP conjugated antibody prior to exposure to chromogen substrate.

Quantification of positive cells by immunohistochemistry

Slides were observed on an Olympus BX 53F microscope. For each specific antibody stain, images from 50 random fields were acquired at 1000x magnification using the software Image Pro version 7 from samples originating from all placentae (ZIKV infected and controls). The number of positive cells were quantified in each of the 50 fields and after segregating the fields to the three conditions ($ZIKV^{+}MIC^{+}$; $ZIKV^{+}MIC^{-}$ and $ZIKV^{-}MIC^{-}$) the mean number of positive cells per field was calculated. All image acquisitions were performed by an individual blinded to the diagnosis associated with the tissue sample. Figures present representative fields to best convey the quantification results.

In situ hybridization

In situ hybridization studies were performed on placentae tissue sections from all cases and controls using a commercial RNA scope Target Probe (catalog #463781; Advanced Cell Diagnostics, USA) that was complementary to sequences 1550-2456 of the ZIKV genome. Pretreatment, hybridization and detection techniques were performed according to manufacturer's protocols. The probe-target complex was revealed by alkaline phosphatase activity on the chromogen substrate nitroblue tetrazolium and bromo-chloroindolyl- phosphate.

Immunofluorescence assay

Paraffin-embedded tissue sections (4 μm) were mounted onto glass slides, deparaffinized in xylene, exposed to decreasing concentrations of ethanol from 100 to 70% and then fully rehydrated in PBS with decreasing alcohol content to 0%. Next, slides were incubated in PBS with 1% bovine serum albumin for 30 min and then permeabilized 30 min in PBS with 0.5% Triton X-100 at room temperature. After washing, slides were co-stained overnight at 4 °C with a 1:200 dilution of a mouse IgG monoclonal anti-Zika NS1 and a rabbit IgG monoclonal anti-human CD163 (Abcam, UK). After washing, sections were incubated with an Alexa 488-conjugated rabbit anti-mouse IgG and Alexa 555-conjugated goat anti-rabbit IgG, diluted 1:200. After washing and mounting, slides were imaged using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Germany).

Molecular diagnosis by RT-PCR

Human serum samples collected on the day of delivery were obtained from six patients and sourced for the isolation of viral RNA using Qiagen RNAEasy. RNA was quantified with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, USA) and purity was evaluated using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) followed by the synthesis of cDNA using First-Strand Synthesis System® (Invitrogen, USA). The amplification reaction was routinely performed by combining the reverse transcription of viral RNA and the subsequent Taq polymerase amplification in a single reaction. The Taqman PCR Master Mix kit (Invitrogen) was used to amplify the the oligonucleotide set utilized targeted the intergenic region of the Membrane/Envelope as described by Lanciotti, 2008 (47). Results were conclusive in two samples.

Molecular diagnosis by PRNT$_{90\%}$

A plaque-reduction neutralization test (PRNT) was performed to detect the presence of neutralizing antibodies against ZIKV in the serum obtained from the six patients mentioned above. Serum samples were incubated at 58 °C for 30 min and then subjected to a series of two-fold dilution beginning from 1:5 to 1:2,560 that were individually incubated with an equal volume containing 100 plaque forming units (PFU) of ZIKV (strain MR 766) at 37 °C. After 1h, the virus-plasma mixture was inoculated onto a confluent monolayer of VERO cells. After an additional hour, inoculum was removed and a semisolid medium (1.4% carboxymethylcellulose in alpha-MEM supplemented with 1% fetal bovine serum) was layered on top of the cells, which were cultured for 5 days before fixation with 4% formaldehyde. Cells were stained with a crystal violet dye solution and the PRNT end-point titers were expressed as the reciprocal of the last serum dilution showing a ≥90% reduction in plaque counts. A PRNT$_{90}$ titer ≥20 was considered positive for the presence of neutralizing antibodies against to ZIKV.

Electron Microscopy analysis

Placental tissue samples were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2), post-fixed with 1% buffered osmium tetroxide, dehydrated in an acetone series (30, 50, 70, 90 and 100%) and embedded in EPON that was polymerized at 60 °C for 3 days. Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate before visualization on a JEOL 1001 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Statistical analysis

Data were analyzed with GraphPad Prism software v 6.0 (GraphPad Software, CA, USA) using non-parametric statistical tests. Significant differences between the analyzed groups were determined using the One-Way ANOVA test with post-hoc Tukey, with a threshold of $P < 0.05$.

Author contributions

KR and MVP designing research studies; LJS, LNM, LDN and LFM collected the material and clinical exams; KR, NGS, PGP and LMH conducting experiments, KR acquiring data; KR, EAP and MVP analyzing data; MVP, JJC, AT and RBO and FBS providing reagents; KR writing the manuscript; DWP and MVP critical reading of the manuscript; all authors agreed with the manuscript.

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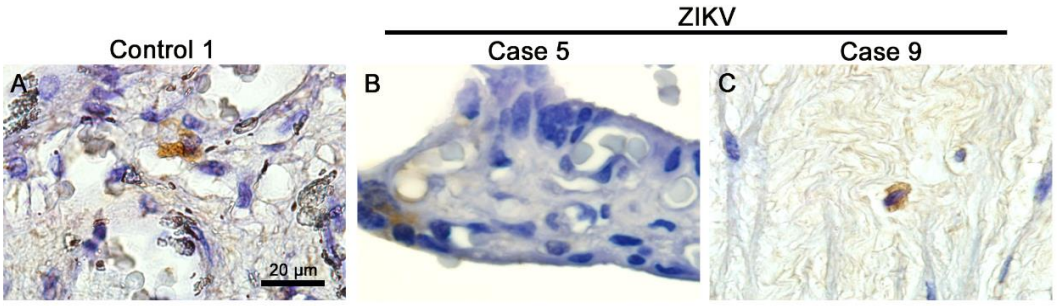
Supplementary Table 1. Description of all histopathological changes found in the patients' HE stained slides and PRNT titers.

	Histopathological changes	PRNT titer
Case 1	Acute and chronic deciduitis, chronic intervillositis, intervillous congestion and dysmorphic villi.	-
Case 2	Edema, hemorrhage, endothelial thickening, immature chorionic villi and chronic deciduitis, extramedullary hemopoiesis, perivascular fibrosis and villositis and stromal fibrosis.	1/20
Case 3	Immature chorionic villi, chronic and acute deciduitis, Hofbauer's cell hyperplasia, villositis and intervillositis	1/20
Case 4	Excessive syncytial *nodes***,** intervillous congestion, fibrinoid necrosis, immature chorionic villi and basal focal chronic villositis.	1/640
Case 5	Chronic deciduitis, immature chorionic villi, Hofbauer's cell hyperplasia and intervillositis	-
Case 6	Endothelial thickening, fibrin areas, infarct, calcification, chronic deciduitis, basal chronic villositis and immature chorionic villi.	1/80
Case7	Focal acute and chronic deciduitis, excessive syncytial *nodes,* intervillous congestion, immature chorionic villi and basal focal chronic villositis.	-
Case 8	Fibrin areas, immature chorionic villi, villositis, acute deciduitis, ischemia from chronic maternal vascular hypoperfusion, villous rarefaction (secondary to ischemia) and hypoplasia.	1/10
Case 9	Endothelial thickening, ischemia from chronic maternal vascular hypoperfusion, vascular congestion, calcification, immature chorionic villi and extramedullary hemopoiesis.	1/20
Case 10	Endothelial thickening, calcification, acute and chronic deciduitis, basal chronic villositis and immature chorionic villi.	-



Supplementary Fig. 1- Detection of T CD4+ Lymphocytes in control and ZIKV-infected placental tissues. **A**) Detection of CD4+ cells by immunohistochemistry in chorionic villi of control placenta. **B-C**) Detection of CD4+ cells by in chorionic villi and decidua of infected placentae.

2.4 **Zika virus infects human placental mast cells and HMC-1 cell line, triggers degranulation, cytokines release and ultrastructural changes (Artigo publicado)**

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Article

Zika Virus Infects Human Placental Mast Cells and the HMC-1 Cell Line, and Triggers Degranulation, Cytokine Release and Ultrastructural Changes

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Abstract: Zika virus (ZIKV) is an emergent arthropod-borne virus whose outbreak in Brazil has brought major public health problems. Infected individuals have different symptoms, including rash and pruritus, which can be relieved by the administration of antiallergics. In the case of pregnant women, ZIKV can cross the placenta and infect the fetus leading to congenital defects. We have identified that mast cells in the placentae of patients who had Zika during pregnancy can be infected. This led to our investigation on the possible role of mast cells during a ZIKV infection, using the HMC-1 cell line. We analyzed their permissiveness to infection, release of mediators and ultrastructural changes. Flow cytometry detection of ZIKV-NS1 expression 24 h post infection in 45.3% of cells showed that HMC-1 cells are permissive to ZIKV infection. Following infection, β-hexosaminidase was measured in the supernatant of the cells with a notable release at 30 min. In addition, an increase in TNF-α, IL-6, IL-10 and VEGF levels were measured at 6 h and 24 h post infection. Lastly, different intracellular changes were observed in an ultrastructural analysis of infected cells. Our findings suggest that mast cells may represent an important source of mediators that can activate other immune cell types during a ZIKV infection, which has the potential to be a major contributor in the spread of the virus in cases of vertical transmission.

Keywords: flavivirus; immune response; inflammatory mediator

1. Introduction

Zika fever is an important *Arbovirus*-caused disease that has surfaced in numerous countries in Asia, Africa and America [1]. The etiological agent of this disease, Zika virus (ZIKV), was initially isolated in 1947 from the blood of sentinel *Rhesus* monkeys during a study on yellow fever transmission

in the Zika forest of Uganda, which gave rise to its name [2,3]. Transmission of the ZIKV is primarily through bites of infected *Aedes* mosquitos, with the most common vectors being *Aedes aegypti* and *Aedes albopictus*, but it can also happen by vertical transmission [4,5]. As a result of vertical transmission, there were alarming cases of Congenital Zika Syndrome, as the virus could cause damage to the placenta, infect placental cells and reach the fetus [6]. A ZIKV particle has a diameter of 25–30 nm and is a member of the *Flaviviridae* family that shares many similarities with other more widely known related viruses such as dengue, West Nile, Japanese encephalitis and yellow fever [4,7]. It has a single-stranded RNA genome with a positive polarity of 11 Kb and encodes a polyprotein precursor that is processed into the structural proteins such as capsid (C), pre-membrane (prM) and envelope (E) along with seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [8,9].

Mast cells are resident immunological cells found abundantly in tissues such as skin, endometrium and placenta that have prominent roles in immunologic reactions [10–13]. Their presence and prevalence in these tissues, along with their proximity to blood vessels, predispose these cells to be among the first immune cells that can be infected by ZIKV after a mosquito bite penetrates the skin. As some of the most frequent symptoms of zika are rash and pruritus, which are relieved by the administration of antiallergic drugs (anti-histamines), this has led us to believe that mast cells can play a role, although not yet elucidated, in the pathogenesis of the disease [14–16]. We hypothesize that it may be one of the cells involved in placental infections, which can directly contribute to vertical transmission.

Although there are no studies in the literature that have investigated the involvement of mast cells in a ZIKV infection to date, mast cells have a proven role in infections by dengue, another *Arbovirus*. Several products originating from mast cells are found at high levels in patients infected by dengue, especially those with plasma leakage [17,18]. While mast cells are permissive to dengue infection, it is most probable that they display a low level of the specific receptors required since the quantity of virus necessary to successfully infect this cell type is always higher than is needed for macrophages and dendritic cells [17,19,20].

HMC-1 cells are a lineage of human mast cells that characteristically express the cytokine receptor c-Kit abundantly and release different cytokines after degranulation stimuli. This cell line possesses the features necessary to serve as an in vitro model for the development of studies on mast cells [21]. HMC-1 has been widely used in studies on degranulation studies, endothelial activation and its interaction with other arboviruses [22–24].

Here, we present our observations on the presence of mast cells in ZIKV-infected human placentae and observed viral replication in these cells. Additionally, we investigated the potential for ZIKV to infect HMC-1 cells as a model system for mast cells and quantified the percentage of infected cells in different MOIs. We further studied the degranulation of these cells after contact/infection with ZIKV by measuring β-hexosaminidase release as well as the expression profiles of TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6), IL-10 (interleukin-10) and VEGF (vascular endothelial growth factor). As a final point, we evaluated the effects of ZIKV infection on the ultrastructure of HMC-1 cells. Together, the findings validate a critical and, to our knowledge, previously unrecognized role for mast cells in the infection and propagation of ZIKV in humans.

2. Materials and Methods

2.1 *Placentae Collection, Patient Clinical History and Ethical Approval*

At delivery, samples from the placentae were collected and fixed in 10% formaldehyde. Samples were collected at the Hospital Plantadores de Cana, Campos dos Goytacazes, RJ, Brazil. As a control, a sample of a full-term placenta from a healthy donor was included.

Case 1: A 23-year-old patient. Symptoms: fever, arthralgia, exanthema and pruritus in the third trimester of gestation. At 38 weeks of gestation, her baby girl was born by cesarean delivery, with 37 cm of cephalic circumference. The mother's IgM serology was positive for Zika. The test for dengue NS1 was negative.

Case 2: A 34-year-old patient. Symptoms: exanthema and pruritus in the third trimester of gestation. Her baby girl was born at term, by cesarean delivery, with 38 weeks of gestation. She presented with a normal 34 cm of cephalic circumference. The mother's IgM serology was positive for Zika.

Patient recruitment and the procedures performed were pre-approved by the Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ (CAEE: 65924217.4.0000.5248) and by the Ethics Committee of Faculty of Campos Medicine/Benedito Pereira Nunes Foundation (CAEE: 65924217.4.3001.5244). The patients were fully informed of the research plans and provided written consent to participate, which included permission to publish all data without identifying information.

2.2 *Histopathology and Histological Detection of Mast Cells in ZIKV Infected Placentae*

All histological processing of the sample was performed as described previously by our group [25]. The histopathological analysis was performed on the images observed and captured by hematoxylin and eosin (H&E) staining. The staining used to highlight the mast cells was Toluidine Blue 1%. Stained specimens were visualized by light microscopy (Olympus, Tokyo, Japan), and digital images were obtained using Image-Pro Plus software version 7.0.

2.3 *Immunofluorescence Assay*

Immunofluorescence was performed as described in Rabelo et al., 2017 [25]. Antibodies were used at a dilution of 1:200 for a mouse monoclonal anti-Zika NS1 IgG (Arigo Biolaboratories, Taiwan, Republic of China), and a rabbit polyclonal antihuman c-Kit IgG (Santa Cruz, Texas, USA). After staining with primary antibodies, sections were incubated with an Alexa 488-conjugated rabbit anti-mouse IgG, Alexa 555-conjugated goat anti-rabbit IgG, or Alexa 555-conjugated goat anti-mouse IgG (ThermoFisher, Waltham, MA, USA). Slides were visualized by fluorescence microscopy (Olympus, Tokyo, Japan), and digital images were obtained using Image-Pro Plus software version 7.0.

2.4 *Immunohistochemistry*

The protocol for immunohistochemistry was described previously by our group [25]. Briefly, the slides were incubated overnight at 4 °C with a 1:200 dilution of the mouse monoclonal antibody IgG antibody against Zika NS1 (Arigo Biolaboratories, Taiwan, Republic of China). Then, sections were maintained with a rabbit anti-mouse IgG conjugated to horseradish peroxidase (Spring Bioscience Corporation, CA, USA) for 40 min at room temperature. We visualized the sections by light microscopy (Olympus, Tokyo, Japan), and digital images were obtained using Image-Pro Plus software version 7.0.

2.5 *Cell Line*

The HMC-1 cell line was kindly provided by Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, NY, USA) and cultured in Iscove's Modified Dulbecco's Medium (IMDM- Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil), 40 U/mL penicillin/streptomycin (Sigma, St. Louis, MS, USA) and 1.2 mM α-thioglycerol (Sigma, St. Louis, MS, USA). Cells were maintained at 37 °C in a humidified incubator at 5% CO_2. Culture media was exchanged every 3–4 days with splitting of cultures at a confluency of 80–90%.

2.6 *ZIKV Viral Stock Productuion*

A primary clinical virus specimen was isolated from a serum sample of a patient from Paraiba. The virus was propagated in a culture of C6/36 *Ae. albopictus* mosquito cells and harvested virus was tittered by the infection of Vero cells (CCL-81) followed by RT-PCRq, which determined a titer of 5.8×10^6 PFU/mL. Copy numbers were assessed by using a standard curve in the RT-PCRq reaction containing 1×10^8 copies/reaction. The oligonucleotide set utilized targeted the intergenic region of the Membrane/Envelope as described by Lanciotti, 2008 [26] (Table 1).

Table 1. Oligonucleotide sets to amplify ZIKV genome.

Genome Position	Region		Sequence
835–857	M/E	sense	TTGGTCATGATACTGCTGATTGC
911–890	M/E	reverse	CCTTCCACAAAGTCCCTATTGC
860–886	M/E	probe	FAM-CGGCATACAGCATCAGGTGCATAGGAG-NFQ

2.7 *ZIKV Infections*

Infections were performed by varying the multiplicity of infection (MOI) at 0.1, 0.2 and 1.0. ZIKV viral particles per host cell. Virus was added to cell culture and incubated for 1 h at 37 °C prior to removal of unattached viral particles and a further incubation of 6 h or 24 h. For a 30 min time point, virus was incubated with cells for 30 min before rinsing and preparation of flow cytometry. As a negative control, cells were incubated in the same conditions with a mock viral stock consisting of a supernatant of non-infected Vero cells.

2.8 *Flow Cytometry Analysis*

The expression of NS1 protein in infected HMC-1 cells was analyzed by flow cytometry. Cells were collected by centrifugation, and suspended in PBS for 30 min, 6 h or 24 h after infection with different MOIs. Approximately 106 cells/well were fixed in 4% formaldehyde for 25 min and permeabilized with 0.05% saponin for 30 min. Next, cells were incubated with a 1:1000 dilution of the mouse monoclonal IgG antibody against ZIKV non-structural protein NS1 (Arigo Biolaboratories, Taiwan, Republic of China) for 1 h at 37 °C before being washed with PBS. This was followed by an incubation with a 1:200 dilution of an Alexa 488-conjugated anti-mouse (Thermo Fisher, Waltham, MA, USA) for 30 min. After washing with PBS, cells were suspended in PBS and applied to a flow cytometer (Facs Calibur; BD Biosciences, San Jose, CA, USA) to measure fluorescence, which was analyzed offline with Summit 6.1 software.

2.9 *Measurement of Mast Cell Degranulation*

Mast cell degranulation was evaluated by measuring the activity of the granule-stored enzyme-β-hexosaminidase that was secreted into the extracellular medium. Cells were infected with MOI 0.1, 0.2 or 1 in 6-well plates (1×10^6/well) for 30 min. Aliquots of the supernatant (15 µl) were transferred to 96-well plates and incubated with 60 µL of substrate (1 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide) in 0.05 M sodium citrate (pH 4.5) for 60 min at 37 °C. In addition, we used 60 µL of substrate solution (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, St. Louis, MS, USA) in 100 mM sodium citrate, pH 4.5) and incubated for 60 min at 37 °C. Reactions were stopped by adding 150 µL of 0.1 M Na_2CO_3-$NaHCO_3$ buffer (pH 10). Enzyme activity was measured as the absorbance at 405 nm. Total β-hexosaminidase activity was determined by releasing all enzyme through lysis with 0.1% Triton X-100 and measuring activity from a 15 µl aliquot. As a positive control for degranulation, we used 20 µg/mL of 48/80 compound (Sigma, St. Louis, MS, USA). The results are presented as the percentage of total β-hexosaminidase content of the cells.

2.10 *ELISA Assays*

The quantity of cytokines and factors released from mast cells by infection with ZIKV was measured by ELISA. Supernatants from HMC-1 cells infected at a MOI of 1 for 30 min, 6 h or 24 h were evaluated for IL-6 (900-T16), IL-10 (900-K21), TNF-α (900-T25) and VEGF (900-K10) with commercial ELISA assay kits (Peprotech Inc. Rocky Hill, NJ, USA), according to the manufacturer's instructions.

2.11 *Transmission Electron Microscopy Procedure*

HMC-1 cells were infected with ZIKV at a MOI of 1 for 30 min or 24 h and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Cells were post-fixed with 1% buffered

osmium tetroxide, dehydrated in an acetone series (30, 50, 70, 90, and 100%) and then embedded in EPON (Electron Microscopy Sciences, Hatfield, PA, USA) through polymerization at 60 °C for 3 days. Ultrathin sections (60–90 nm) were contrasted with uranyl acetate and lead citrate before visualization using a JEOL 1001 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

2.1 *Statistical Analysis*

2

Data were analyzed in GraphPad Prism software v 6.0 (GraphPad Software, San Diego, CA, USA) using non-parametric statistical tests. Significant differences between the analyzed groups were determined using the Mann–Whitney test with a threshold of $p < 0.05$.

3 Results

3.1 *Detection of Mast Cells, Histopathology and ZIKV Replication in Placental Infected Tissues*

First, we evaluated the presence of mast cells in the placentae of ZIKV infected women during pregnancy in comparison to a non-infected control sample. To detect mast cells, we performed immunohistochemistry with a Toluidine Blue stain and identified these cells in placental sections of these patients by the prominent purple coloration (Figure 1A–C, arrows). Next, fluorescence microscopy images (Figure 1D–F) were used to identify cells that displayed both the mast cell marker c-Kit (red) and ZIKV NS1 protein (green). As expected, no evidence of ZIKV NS1 protein was observed in control placenta (Figure 1D). In constrast, dually labeled cells were readily observed in placenta from both ZIKV seropostive patients (Figure 1E,F), which suggested that these cells were infected and supported virus replication (Figure 1E,F). To examine the histopathological aspects, H&E stainging was used to identify maternal portions (basal decidua) and fetal portions (chorionic villi), which were normal in the control placenta (Figure 1G). Within the placentae from the ZIKV infected patients, case 1 presented areas with immature chorionic villi, chronic villositis and chronic deciduitis with lymphocytes in chorionic villi and decidua (Figure 1H). The placenta from case 2 showed intervillitis with lymphocytes in the intevillous space and immature chorionic villi (Figure 1I). To extentd the search for cells supporting ZIKV replication, immunohistochemistry was used to provide broad staining of NS1 protein both in the maternal and fetal portions of the placentae. Again, the control, non-infected samples showed no reactivity against NS1. Within placentae from infected mothers, extensive reactivity was seen in not only immune cells, but also trophoblasts and decidual cells suggesting that they are also permissive to infection (Figure 1K,L).

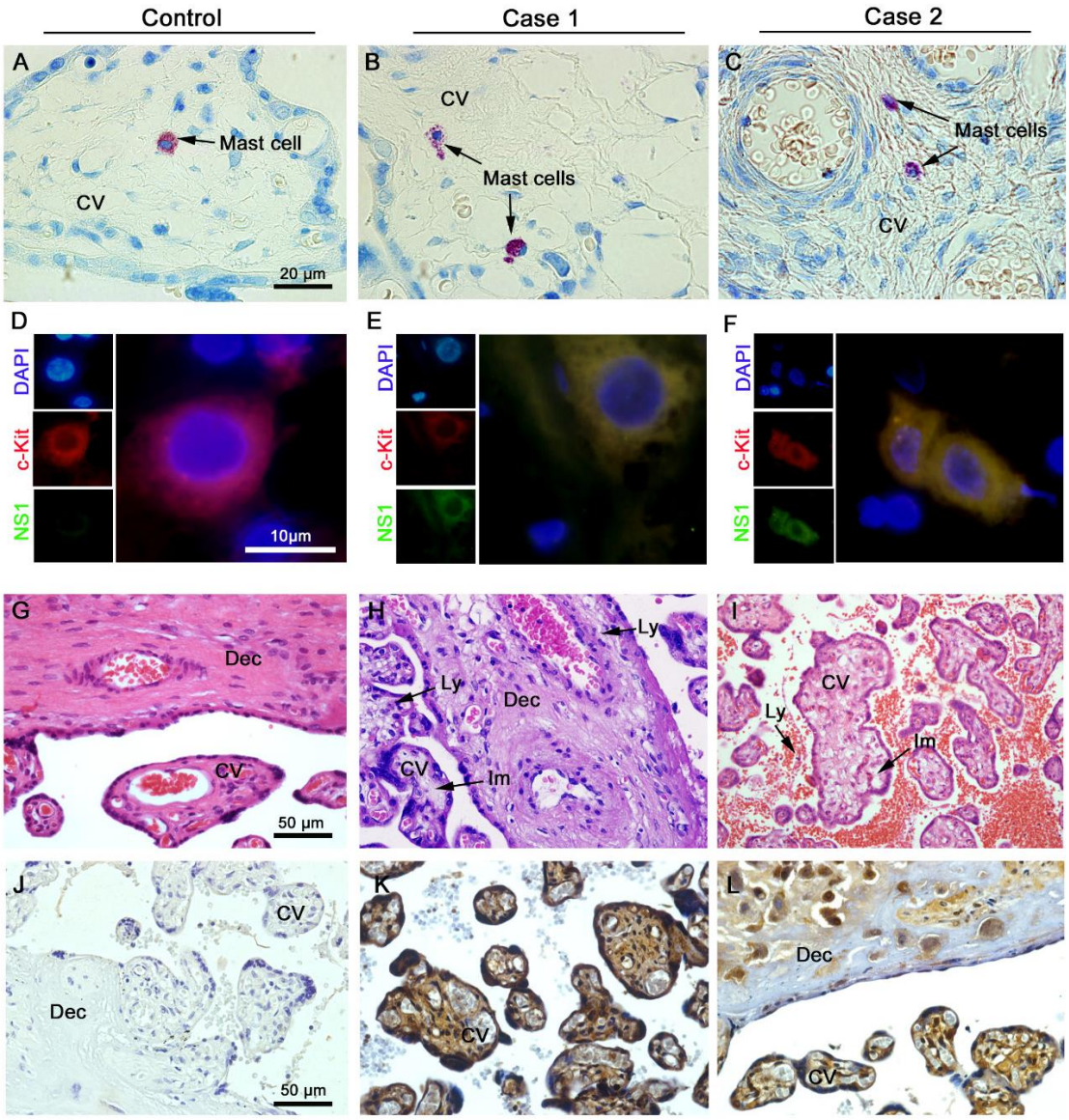

Figure 1. Detection of ZIKV infected mast cells in placental tissue from seropositve mothers. Placentae were collected from mothers infected or not with ZIKV immediately after childbirth and preserved in formadehyde. (**A–C**) Brightfield images of sections stained with Toluidine Blue showing metacromatic granules (purple, arrows) in mast calls. (**D–F**) Immunofluorescent images of DAPI (nuclei; blue), c-Kit (mast cell marker; red) and NS1 (ZIKV marker; green) showing ZIKV infected mast cells with both red and green fluorscence. No NS1 antigen was observed in any sections from the control placenta. The histopathological analysis of the H&E stained placentae showed normal aspects in decidua and chorionic villi within the control placenta (**G**), whereas infected placentae showed areas with lymphocytic infiltrates and immature chorionic villi (**H**,**I**). Detection of ZIKV NS1 protein by immunohistochemisty did not identify any positive cells in control placentae (**J**). Numerous cells positive for NS1 were detected in placentae from infected mothers, in both maternal and fetal portions (**K–L**). CV, chorionic villi; Dec, decidua; Im, immature chorionic villi; Ly, lymphocytes.

3.2 *Infection Rate of ZIKV at Different MOIs*

After observing that placental mast cells were infected with ZIKV during a natural infection, the susceptibility to ZIKV entry and permissiveness to its replication was evaluated using the HMC-1 cell line under controlled conditions. Cells were exposed to three different MOIs (0.1, 0.2 and 1) of virus or an equal volume of mock as a control to determine conditions of infections. A mock viral stock was

generated from supernatants of Vero cells that were not exposed to ZIKV as a control. The percentage of cells infected by ZIKV was determined by counting the number of cells displaying the fluorescent detection of NS1, a protein that is present only after viral replication, by flow cytometry. Cells were either incubated with virus or mock for 30 min and processed for analysis, or for 1 h with a subsequent incubation for 6 h or 24 h. NS1 was detected under all conditions (Figure 2A), even after 30 min, which suggests that ZIKV can rapidly enter cells and begin replication. Considering that the percentage of cells was nearly equivalent across the three MOIs at 30 min, the results further suggest that only a subset of cells were susceptible to rapid infection. By increasing the virus binding and entry time to 1 h, followed by a 6 h incubation, the percent of cells infected increased with a maximum percent observed with a MOI of 1. A slight increase in the percentage of cells was measured when the post-infection incubation increased to 24 h. Averaged histograms of the three conditions (Figure 2B–D) show a nearly equivalent low background from the mock and the highest levels of infection with a MOI of 1 in 6 h and 24 h, with a mean of 40.10 ± 4.81 and 45.30 ± 3.44% of infected cells in three independent experiments, respectively.

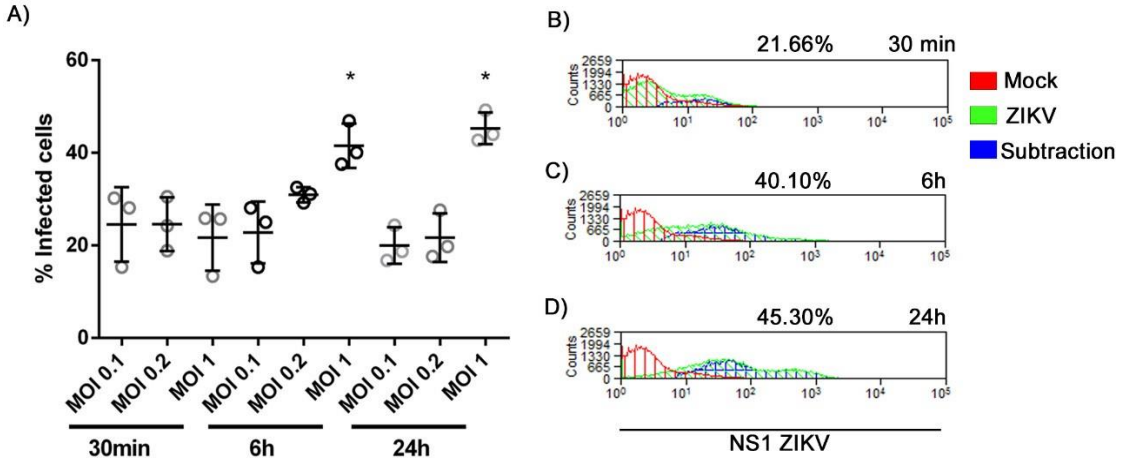

Figure 2. Percentage of HMC-1 cells infected with different MOIs of ZIKV. HMC-1 cells were incubated with ZIKV at MOIs of 0.1, 0.2 or 1 for 30 min and prepared for flow cytometry, or 1 h followed by 6 h or 24 h incubation before analysis. Cells were permeabilized, fixed and stained with the mouse monoclonal IgG antibody against ZIKV non-structural protein NS1 followed by incubation with the Alexa 488-conjugated anti-mouse. Panel (**A**) presents the individual percentages of HMC-1 cells expressing the NS1 protein under the different conditions from three independent experiments. Averaged histograms from the experiments with an MOI of 1 are shown in (**B**) 30 min, (**C**) 1 h with 6 h and (**D**) 1 h with 24 h infection. For negative control, cells were incubated with mock viral stocks. * Statistically significant differences between groups (same time of infection) assessed by a Mann–Whitney test ($p < 0.05$).

3.3 *ZIKV Interaction Induces Degranulation*

The results from the infection of mast cells by ZIKV suggested that the response of HMC–1 could be contributing to the observations. We chose to explore the activation and degranulation of mast cells by β-hexosaminidase, a resident enzyme released in response to degranulation. Initially, flow cytometry was used to analyze the percentage of cells that display degranulation following incubations of HMC-1 cells with ZIKV at different MOIs for different times. After a 30 min incubation, all three MOIs showed similar percentages (Figure 3A). The percentage of cells decreased following a 6 h incubation and returned to the levels of mock infections after a 24 h incubation suggesting that, at the later time points, the granulosome recuperated or the released enzyme lost activity.

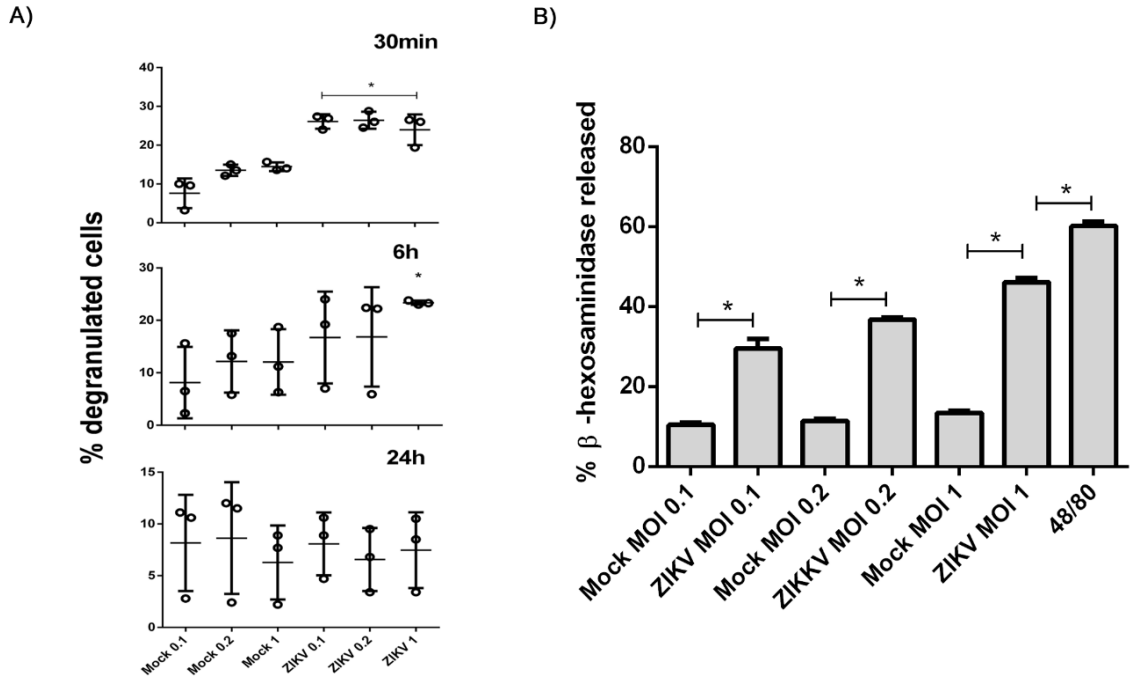

Figure 3. Kinetics of mast cell degranulation after interaction with ZIKV. (**A**) Percentage of degranulated cells after incubation with different MOIs of ZIKV in 30 min, 6 h and 24 h by flow cytometry. (**B**) Percentage of β-hexosaminidase release with different MOIs of ZIKV after 30 min. The synthetic compound 48/80 was used to elicite mast cell degranulation. * Statistically significant differences between groups (same time of infection) assessed by a Mann–Whitney test ($p < 0.05$). Data represent the mean of duplicate values for each sample, in three independent experiments.

To evaluate the early kinetics of mast cell activation, the amount of β-hexosaminidase, normalized to the total cellular β-hexosaminidase, was measured at 30 min for each of the MOIs (Figure 3B). Despite the β-hexosaminidase levels not reaching the percentage of the cells stimulated with the synthetic compound 48/80, the release was gradually increased according to the amount of viral particles, suggesting that the activation of these cells actually occurs due to adsorption of the virus to cell receptors.

3.4 *ZIKV Led to Release of Cytokines and VEGF*

To analyze the release of the cytokines TNF- α, IL-6, and IL-10, along with VEGF, during infection with ZIKV, we performed ELISAs on the supernatant of mast cells activated with 30 min of contact with the virus as well as the extracellular levels produced by 1 h of virus presence and an incubation of 6 or 24 h. After the shortest interaction time, the levels of TNF-α, IL-6 and IL-10 increased greater in response to exposure to the control than with ZIKV (Figure 4). The levels of VEGF were nearly equal. The levels of the cytokines and VEGF in the supernatant were significantly greater after a hour incubation with ZIKV stocks with an additional 6 h incubation than the mock stocks. This difference grew with the increase in the secondary incubation time to 24 h although the absolute levels of these cytokines and VEGF were lower compared to 6 h. The observed release of TNF-α, IL-6, IL-10 and VEGF at 30 min suggested that they responded to a range of external stimuli. Meanwhile, the elevated levels of these mediators 6 h or 24 h after the infection with ZIKV infection suggests a stimulation in expression and release of these mediators after infection.

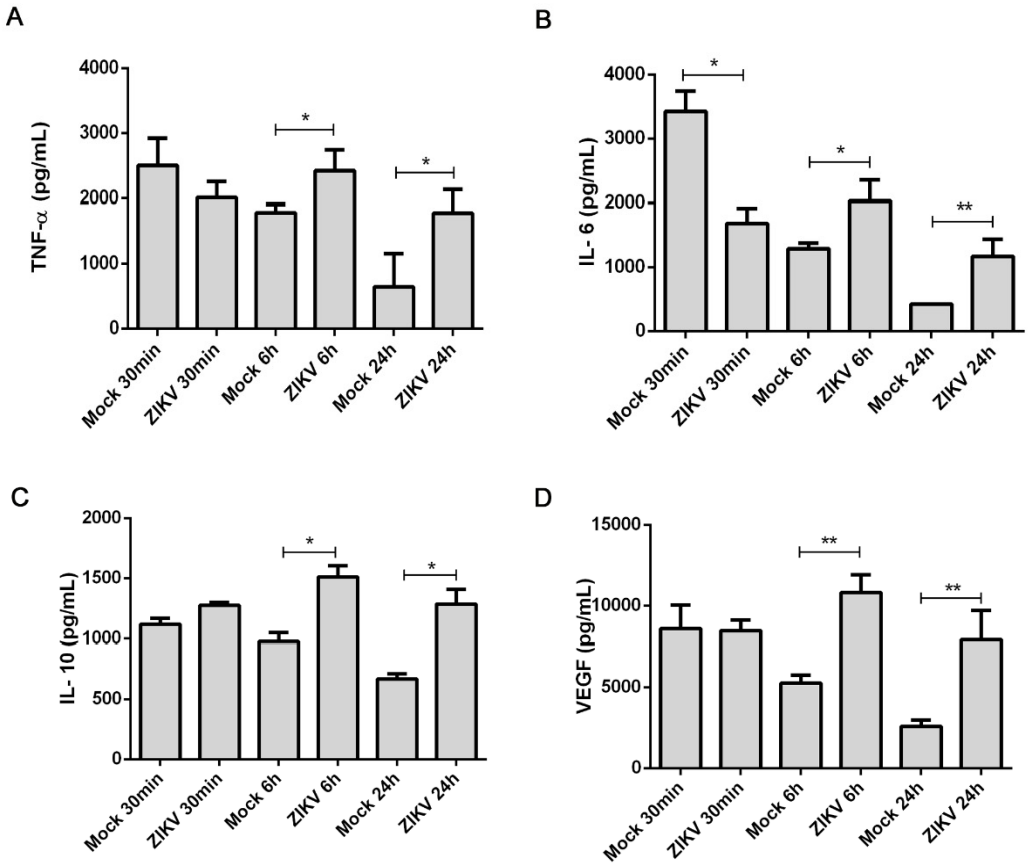

Figure 4. Cytokine and VEGF release by HMC-1 cells in response to ZIKV interactions. The supernatants of HCM-1 cells were collected after incubation with ZIKV or mock viral stocks for 30 min or following a 1 h incubation with an additional 6 or 24 h incubation. Commercial ELISAs were used to measure the level of released (**A**) TNF-α, (**B**) IL-6, (**C**) IL-10 levels and (**D**) VEGF. Data represent the mean of triplicate values for each sample obtained from three independet experiments. * Statistically significant differences assessed by a Mann–Whitney test ($p < 0.05$).

3.5 *Ultrastructural Changes Caused by ZIKV Infection*

To explore changes to aspects of the ultrastrutucture of mast cells in response to ZIKV infections, an infection with an MOI of 1 was used for the best conditions of infection as well as activation and degranulation of HMC-1. As a control for the analysis, the ultrastructure of cells incubated with the mock viral stock for 30 min was evaluted. Representative cells presented normal aspects for a mast cell in terms of the formation of the nucleus, and the volume of the mitochondria and normal endoplasmic reticulum with a high density of granules (Figure 5A–C). While cells incubated with the ZIKV for 30 min have a lower rate of infection, our previous data show they are at the optimal moment of adsorption and trigger degranulation. The ultrastructure of representative cells shows a decrease in cellular granules (Figure 5D–F), with no other major alterations. After 24 h incubation with the mock viral stock, we observed that the mastocytes continued to have a high density of granules, endoplasmic reticulum with closed cisterns, and mitochondria with some structural alterations, such as swollen and ruptured (Figure 5G–I). After the same period of incubation, the infected mast cells presented various organelle alterations observed as the formation of numerous vesicles, dilated endoplasmic reticulum cisterns, swollen mitochondria, ruptures in cellular membranes and, in some cells, the absence of a nucleus suggesting that a subset of cells may no longer be viable (Figure 5J–K). In several instances, the presence of viral-like particles were detected that match with the size of a ZIKV particle (Figure 5L).

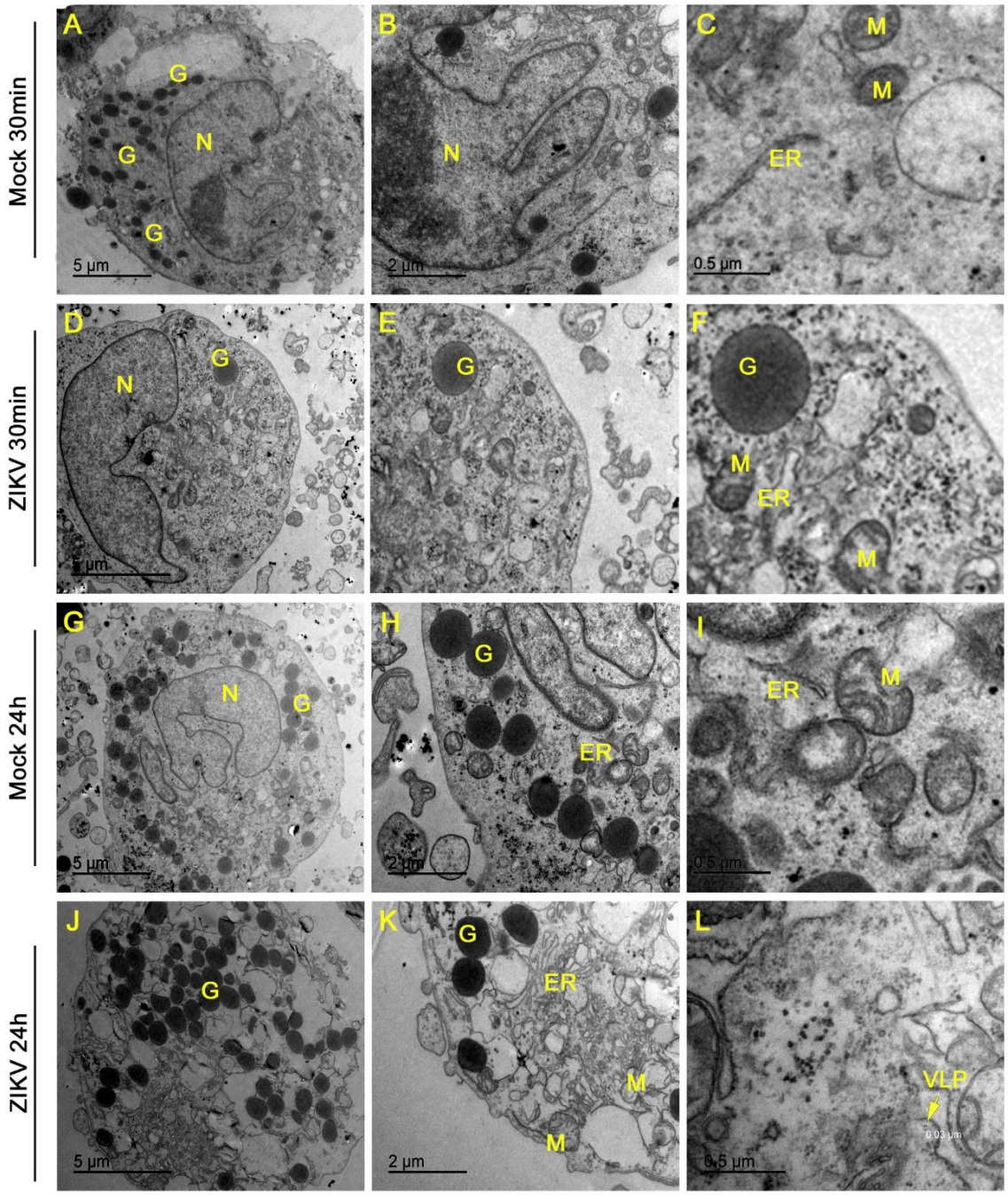

Figure 5. Ultrastructural changes in HMC-1 mast cells infected with ZIKV. HCM-1 cells were exposed to ZIKV or mock for 30 min or 1 h with a post 24 h incubation before processing and imaging of ultrathin sections by electron microscopy. (**A–C**) Control HMC-1s incubated with mock for 30 min. (**D–F**) An HMC-1 cell incubated with ZIKV for 30 min with decreased granules. (**G–I**) An HMC-1 cell incubated with mock for 24 h with a high density of granules. (**J–L**) HMC-1 cell infected with ZIKV for 24 h. Panel L shows a virus-like particle (VLP) with a diameter of approximately 30 nm, consistent with ZIKV. Granules (**G**), nucleus (**N**), mitochondria (**M**) and endoplasmic reticulum (ER).

4 Discussion

Mast cells have an important function in developing an inflammatory process and are present in a variety of tissues such as skin and mucous membranes that include the placenta. However, there have been no studies that have investigated the role of mast cells in ZIKV infection and its

pathogenesis. Many studies have described a permissiveness and replication of ZIKV in different placental and immune cells [25,27,28]. These descriptions are extensive in relation to Hofbauer cells and deciduous macrophages [29–31]. Here, we report for the first time the detection of virus in vivo in mast cells present in placental tissue from two women seropositive for a ZIKV infection through the NS1 protein of Zika. Mast cells are resident cells in the endometrium and placenta, and it is believed that they can play multiple roles from implantation to placental immune response during pregnancy, including trophoblastic migration and angiogenesis [32,33]. The implications of ZIKV infections in placental mast cells could have some importance in understanding the inflammatory process and vertical transmission.

Based on the in situ results, we performed a series of experiments in vitro using the HMC-1 cell line as a model system for mast cells to unveil aspects of their interactions and reaction to infections by ZIKV. First, we observed by a flow cytometer analysis that the HMC-1 cells are able to support the entry of the virus as well as its rapid replication within 30 min. Replication was inferred by the detection of the NS1 protein of Zika, which is a non-structural protein that is not a constituent of the virus particle and is only present after its synthesis at the time of replication [9]. It is known that mast cells have the requisite receptors, such as FcGR, HSP70 and others, that could mediate the entry of ZIKV, and also mediate the entry of other arboviruses like dengue, as well as being involved in the transduction signals for the degranulation cascade [21,34].

One of the most abundant proteases present in mast cell granules widely used to assess degranulation is β-hexosaminidase, a glycolytic enzyme that is released into the tissues and triggers typical reactions in allergy and inflammatory responses [35]. We used the quantification of β-hexosaminidase in cell supernatants as a measurement of mast cell degranulation as a result of incubations with ZIKV. We used the synthetic compound 48/80, which is a standard degranulator, to elicite β-hexosaminidase release by HMC-1 cells [36]. There was a significant increase in the release of β-hexosaminidase by the HMC-1 cell line after contact with ZIKV, which was only detected at 30 min, which leads us to believe that viral adsorption is a stimulus for degranulation. The time frame of 30 min is consistent with that of the adsorption and internalization of flavivirus particles, which occurs rapidly in 13 to 15 min as observed for the intracellular localization of DENV particles [37]. In MOI 1, β-hexosaminidase levels were near that of the positive control with 48/80. Degranulation, detected by the release of β-hexosaminidase, has been associated with the injection of DENV in other studies [19]. The cleavage of some substrates of this enzyme has been associated with NKT cell differentiation, and the high activity of β-hexosaminidase has already been observed in placental dysfunction [35,38]. In addition to the enzymes released during degranulation, mast cells are responsible for the production and release of different pro-inflammatory cytokines. We evaluated the production of TNF-α, IL-6 and IL-10 at different times from viral adsorption to 6 h and 24 h post infection. At the moment of initial contact of the mast cells with the mock or the virus, there was a release of these cytokines and VEGF, which is consistent with mast cells having internal stores that are primed for release in response to a stimulus. As the supernatant of Vero cells (mock) has a rich secretion of proteins, this stimulus appears to have been sufficient for the release within 30 min. However, at the end of other incubation times, the mock viral stock controls were associated with low secretion levels of these mediators, which contrasted with the ZIKV infected cells. There was a significant increase in levels of both cytokines at 6 h, which would be expected to generate an environment conducive to the recruitment and differentiation of other immune cells. TNF-α is produced for optimal defense against pathogens in inflammation resolution and orchestrates the tissue recruitment of immune cells and promotes tissue remodeling and destruction [39]. IL-6 is a cytokine with a crucial role in inflammation. It also leads to recruitment and differentiation of mast cells, as well as monocytes, CD4+ and CD8+ T cells, and B lymphocytes, and it stimulates the production of VEGF by fibroblasts. IL-6 expression affects the homeostatic processes that is related to tissue injury and activation of stress-related responses [40–43]. Despite being an anti-inflammatory cytokine, the expression of IL-10 was increased in ZIKV-infected HMC-1 cells, which corroborates what was observed in another study, in the serum of Zika positive

patients [44]. Moreover, it is a cytokine normally produced by triggered mast cells, which leads to activation of other mast cells and is present in allergic responses [45,46]. In support of our findings, an increase in cytokines related to the inflammatory environment in placental ZIKV infection has already been observed in another study performed by our group, with an increase in TNF-α and the VEGFR-2 receptor [28]. TNF-α combined with VEGF were similarly related to vascular placental dysfunction, leading to plasma overflow and preeclampsia [47]. The stabilization of mast cells can decrease their response and minimize the severity in dengue, which is related to the release of VEGF and vascular permeability [48].

The ultrastructural changes that occur in the infection can be quite enlightening in relation to the processes that the cell undergoes against the pathogen. We observed degranulation of HMC-1 after 30 min of contact with the virus, but the alterations in organelles were only evident 24 h after infection. The changes caused by ZIKV were already observed in placental cells, and are consistent with those that occur in DENV, even in other cell types [25,49,50]. These changes suggest damage, mainly to mitochondria and the endoplasmic reticulum, which could impinge on the energy and protein production machinery that are necessary for viral replication. In addition, we detected the presence of virus-like particles, with the size expected for ZIKV particles, ~ 30 nm, which confirms the permittivity and ability of mast cells to replicate the virus. These observations, together with the characteristics of mast cells as an immune system component, would suggest that they would be capable of circulating throughout an affected organism, or being resident in the tissue could be responsible for cell-to-cell infection that could underlie vertical transmission.

5 Conclusions

Our data serves as evidence that mast cells are permissive to ZIKV infection, since a non-structural protein, NS1, was detected 24 h post infection. ZIKV can induce degranulation on its first contact and can produce cytokines and VEGF both short term and over a few hours of infection. This response of mast cells can facilitate the installation of a pro-inflammatory environment in the sites where these cells are found, such as in mucous membranes like the placenta. In addition, the fact that they can support the replication of the virus in the human placenta suggests that this type of cell may contribute to vertical transmission. Further studies are needed to fully elucidate the role of mast cells in ZIKV infection.

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2.5 **Zika virus induces oxidative stress and changes the ultrastructure in HTR-8/SVneo, a placental cell line (Artigo submetido**)

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Zika virus induces oxidative stress and changes the ultrastructure in HTR8-SVneo, a placental cell line

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Abstract

Zika virus (ZIKV) is an arbovirus, which can be diffused by vertical transmission causing alarming reports of Congenital Zika Syndrome cases associated with infection. Despite its importance and the studies developed, the pathogenesis of this disease has not yet been clarified. In this work, we investigated the consequences that ZIKV infection can develop on the placental trophoblastic cell lineage, HTR-8/SVneo. We standardized an infection protocol, using different virus MOIs (0.1, 0.2 or 1) per 24h or Mock (negative control), evaluating the number of cells infected by immunofluorescence and flow cytometry. Results by immunofluorescence showed that ZIKV could infect HTR-8/SVneo, and in the infection with MOI 1 we detected a higher intensity of NS1 protein staining in relation to other MOIs, and this was the best condition, confirmed by flow cytometry, which induced the highest percentage of infected cells (44.2%). The ultrastructure of infected cells presented mitochondrial alterations, which were smaller and with loss of mitochondrial crests, endoplasmic reticulum with less dilated cisterns and the presence of many prolongations and vesicles secretion, compared to the Mock. The infected cells showed a significant increase in the activity of the antioxidant enzyme Superoxide Dismutase, and the reactive species Malondialdehyde and Nitric Oxide (1.29±0.41 U/mg protein, 0.01±0.001 nmol/mg protein and 95.68±52.01μmol/ml) compared to the control (0.21±0.11 U/mg protein, 0.003±0.002 nmol/mg protein and 48.79±9.18μmol/ml), respectively. On the other hand, the enzymatic activity of Catalase was lower in ZIKV infected cells (5.1 ± 3.8 U/mg of protein) compared to the control (33.88 ± 30.0 U/mg of protein), due to its rapid consumption and depletion in infection. These results will help to better understand the pathogenesis of the disease, as they may elucidate the consequences of infection in placental cells.

Keywords: flavivirus, HTR-8/SVneo, mitochondria, reactive species

Introduction

Zika fever is an arbovirosis, transmitted mainly by *Aedes* mosquitoes that are vectors for the Zika virus (ZIKV), but there is also the possibility of transmission through sexual contact, blood transfusion and vertical transmission, with alarming reports of Congenital Zika Syndrome (CZS) cases associated with infection. The CZS consists of a spectrum of congenital failures with pathological manifestations of the central nervous system. These include: microcephaly, decreased cerebral volume, ventriculomegaly, calcifications, pyramidal and extrapyramidal signs, that cause paralysis or muscle pairs, ocular lesions of coriorectal atrophy, focal pigmentation of the retina and other alterations [1]. The etiological agent of this illness is approximately 25–50 nm and shares many structural similarities with other flaviviruses such as Dengue, Yellow Fever, West Nile and Japanese encephalitis [2]. The ZIKV genome encodes a polyprotein precursor, which is processed into the structural proteins: capsid (C), pre-membrane (prM), and envelope (E); and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [3].

ZIKV crosses the placenta and infect the fetus, in this way, different groups have been trying to clarify the mechanism of placental transposition and their effects [4–6]. Despite the onslaughts, the pathogenesis of this disease has not yet been fully elucidated. Investigations involving the main pathogenesis mechanisms, the oxidative stress, is a gap to be filled in relation to ZIKV infection, mainly in placental cells. Oxidative stress occurs when the homeostatic balance of production and neutralization of reactive oxygen species (ROS) is misplaced in the cell, leading to anomalous state and inducing harmful changes to the cellular microenvironment. Infections with other flaviviruses, such as dengue, trigger oxidative stress, which affects both cell metabolism and the life cycle of the virus itself [7]. In other cell types, the oxidative stress caused by ZIKV leads to changes in the translation of the RNA produced by the cell, consequently to the assembly of large aggregates composed of the stopped translation pre-initiation complexes, called stress granules, in addition to triggering the process cell death [8,9].

In vitro studies have controlled conditions and can be a great ally in the development of investigations into the effects of viral infection on placental cells. The HTR-8/SVneo cell lineage was developed from human trophoblastic cells that were transfected with the T antigen of the simian virus 40 (SV40). The transfection gives the cell an unlimited dividing capacity, while maintaining the characteristics of human trophoblastic cells [10]. HTR-8/SVneo cells have already been tested in two studies on susceptibility and infection by ZIKV, which are suitable for both viral entry and replication [11,12]. For this reason, the HTR-8/SVneo cell line is considered an appropriate model for *in vitro* studies on the maternal-fetal interface.

In this work, we investigate the ultrastructural changes and the oxidative stress caused by ZIKV in human trophoblastic cells (HTR-8/SVneo).

Material and Methods

Cell culture

The HTR-8/SVneo trophoblastic cells (ATCC, USA) were cultivated in Roswell Park Memorial Institute Medium (RPMI-1640) (SIGMA, USA) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Brazil). Cells were maintained at 37 °C and under humid atmosphere with 5% CO_2. The cell monolayer was washed every 3 days with phosphate buffered saline solution (PBS) (pH 7.4). For the passage of cells, the monolayer was washed with PBS and dissociated with 0.25% trypsin solution (Gibco, USA). In all experiments, cells were infected in a maximum of four cell passages.

Virus and infection assays

The virus used in this work was the ZIKV PB81 strain- isolated from a patient from Paraíba, Brazil, cultivated in C6/36 cells and in Vero CCL81 cells. The viral titre was measured by RT-PCR, Gene Bank KX280026. Virus stocks were maintained at −80 °C until used. Cells were plated in 10^6 confluency/well 24h before infection. The monolayer was infected with the ZIKV at an MOI of 0.1, 0.2 or 1 or with the same volume of Mock (control), and wells were incubated at 37 °C for 1 h with gentle rocking. After, the medium was changed by a medium complemented with 10% FBS, and cultures were maintained for a further 24 h.

Indirect immunofluorescence analysis

Twenty four hours following infection, cell monolayers were washed with 0.1 M phosphate buffer pH 7.4 (PB), fixed with 4% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.6% of saponin in PBS for 10 minutes and blocked with 1% bovine serum albumin (BSA) for 20 minutes. For detection of the ZIKV NS1 protein, cells were incubated with an anti-human monoclonal Zika NS1 (Arigo) (diluted 1:300) for 1 hour at 37°C. Cells were washed and incubated with anti-mouse Alexa 488 (ThermoFisher, USA) in the same conditions. Slides were assembled with ProLong Gold Antifade Mountant (ThermoFisher) and cells were visualized in fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis

Expression of NS1 ZIKV protein in infected HTR-8/SVNeo, was analyzed by flow cytometry. Cells were trypsined from the wells and suspended in PBS 24 h after infection. Approximately 10^6 cells/well were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.05% saponin for 30 min. Subsequently, cells were incubated with anti-human monoclonal Zika NS1 (Arigo) (diluted 1:1000) for 1 h at 37 °C, washed in PBS, followed by incubation with anti-mouse or anti-rabbit Alexa 488 (ThermoFisher, USA) (1:200) for 30 min. Cells were suspended in PBS, read in the flow cytometer CytoFlex (Beckman, USA) and analyzed offline in Summit 6.1 software.

Transmission Electron Microscopy procedure

Cells were trypsinized and fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2), post-fixed with 1% buffered osmium tetroxide, dehydrated in an acetone series (30, 50, 70, 90, and 100%), and embedded in EPON (Electron Microscopy Sciences, PA, USA) polymerized at 60°C for 3 days. Ultrathin sections (60–90 nm) were contrasted with uranyl acetate and lead citrate and were visualized using a JEOL 1001 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Oxidative Stress Analyses

Cells were homogenized in 200 µl of potassium phosphate buffer + EDTA (KPE) (pH 7.5), centrifuged at 600 g for 10 minutes at 4°C, the supernatant collected and the pellet lysate discarded. At the end, the samples were stored at -80º C until the time of the biochemical analyses. Superoxide dismutase (SOD) activity was assayed by monitoring epinefrin (Sigma-Aldrich, USA) inhibition auto-peroxidation. Glycine buffer (pH 10.2) was used to measure SOD activity and the samples were arranged in three different volumes (1 µl, 2 µl and 3 µl). Then 193 µl of glycine buffer, 2 µl of catalase, and 4 µl of epinephrine were added, and the reading was immediately taken in a spectrophotometer (SpectraMax M5 - Molecular Devices) at 480 nm. As a control, a blank was made where the free oxidation of the epinephrine was evaluated without the samples. Catalase (CAT) activity was measured by a decrease of H_2O_2 (Sigma) rate, and concentrations were monitored. For this assay, the "Mix" was prepared, containing 25 ml of distilled water and 40 µl of hydrogen peroxide. Subsequently, 1 µl of sample was added for 99 µl of MIX. The samples were read in a spectrophotometer in absorbance of 240 nm, using a UV plate. After reading, the values were expressed in U / mg protein. As an index of oxidative damage induced by lipid peroxidation, we used thiobarbituric acid reactive substances (TBARS) (EMD Millipore, USA) method to analyze malondialdehyde (MDA) products during an acid-heating reaction. 100 µl of supernatant was collected and 150 µl of thiobarbituric acid (TBA) was added, the samples were heated in a dry water bath at 95 ºC for 10 minutes. MDA levels were determined by absorbance at 532 nm, and expressed as nmol/mg protein. Nitric Oxide quantification was performed by homogenizing the Griess reagent (Sigma) under light. We then plated 100 µl of sample and 100 µl of griess reagent, incubating for 10 minutes. The reading was made at 540 nm, expressed as µmol/mg protein.

Statistical analysis

Data were analyzed with GraphPad Prism software v 6.0 (GraphPad Software, CA, USA) using non-parametric statistical tests. Significant differences between the analyzed groups were determined using the One-Way ANOVA test with post-hoc Tukey, with a threshold of $P < 0.05$.

Results

Primarily, we evaluated the susceptibility and permissiveness of HTR-8/SVneo, and the best condition of infection to perform our experiments. Cells were infected with ZIKV or incubated with Mock (control) at three different MOIS (0.1, 0.2 and 1), in order to determine an ideal condition of infection. The ZIKV NS1 was detected by immunofluorescence in all conditions, however, with MOI 1, the number of cells that were positive and with better labeling intensity were higher than others (Figure 1A). Supporting these results, in the flow cytometry analysis, the highest percentage of infected cells was with MOI 1, with the mean of 62.53% in three independent experiments, rather than 32.43% and 39.67% with MOI 0.1 and 0.2, respectively (Figure 1B-C).

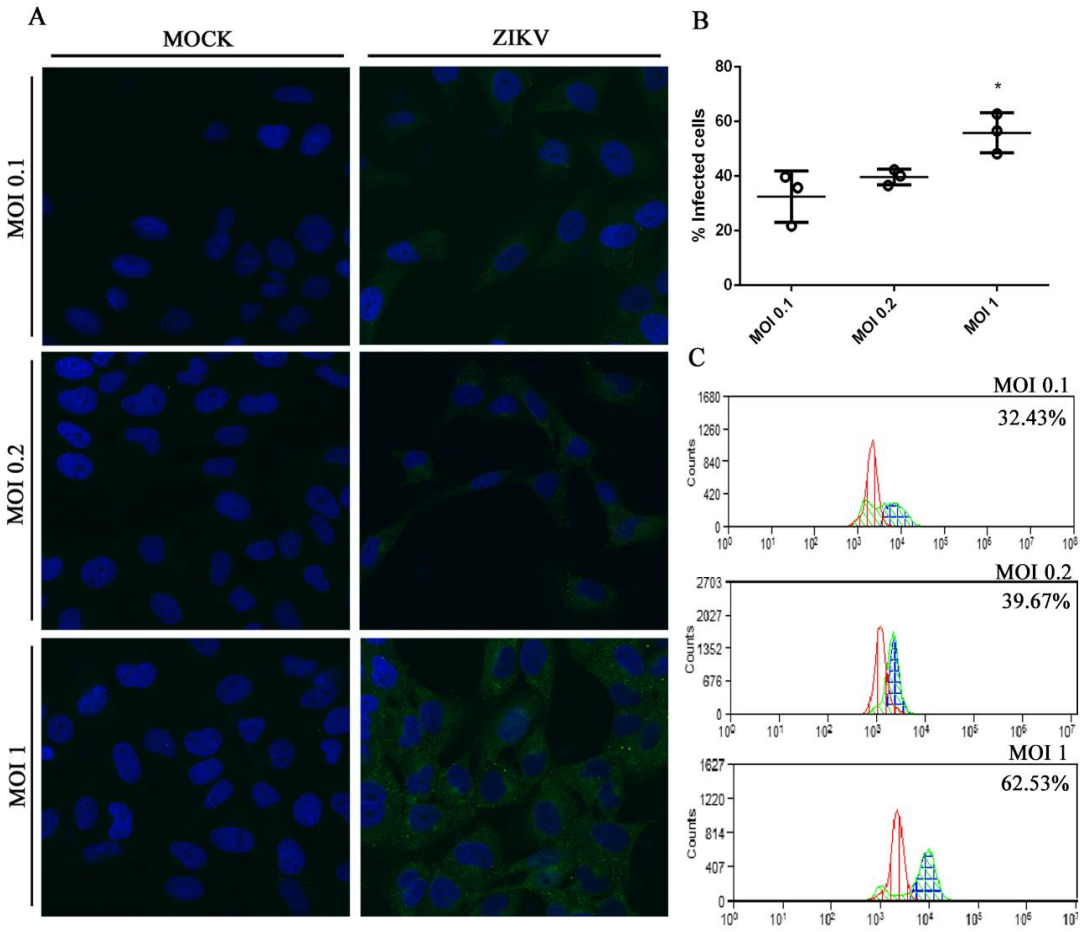

Figure 1. Efficiency of HTR-8/SVneo cells infected with different MOIs of ZIKV evaluated by indirect immunofluorescence and flow cytometry. A) Cells expressing the ZIKV NS1 protein after infection with MOI 0.1, 0.2 or 1. The number of positive cells and the labeling intensity was higher in MOI 1. B) Percentage of HTR-8/SVneo cells expressing the NS1 protein after infection with MOI 0.1, 0.2 and 1 of ZIKV. The better infection condition led to 62.53% of infected cells (with MOI 1). C) Histograms are representative of three independent experiments. For negative control, cells were incubated with Mock. Cells were permeabilized, fixed and treated with the mouse monoclonal IgG antibody against

ZIKV non-structural protein NS1 followed by incubation with the Alexa 488-conjugated anti-mouse. Asteriskis indicates statistically significant differences between groups by a Mann-Whitney test ($p < 0.05$).

We used transmission electron microscopy to ascertain ultrastructure morphological changes caused by ZIKV 24 hours after infection. The aspect of MOCK incubated cells were considered with normal aspects, with nucleus and mitochondria without modifications and endoplasmic reticulum with some dilated cisterns, common in cells with higher protein production, as expected for trophoblastic cells (Figure 2A-B). In contrast, HTR-8/SVneo cells infected with ZIKV showed prolongations, a characteristic of activated cells, secretion of vesicles, mitochondria with loss of mitochondrial crest and abundant endoplasmic reticulum with less dilated cisterns (Figure 2C-D).

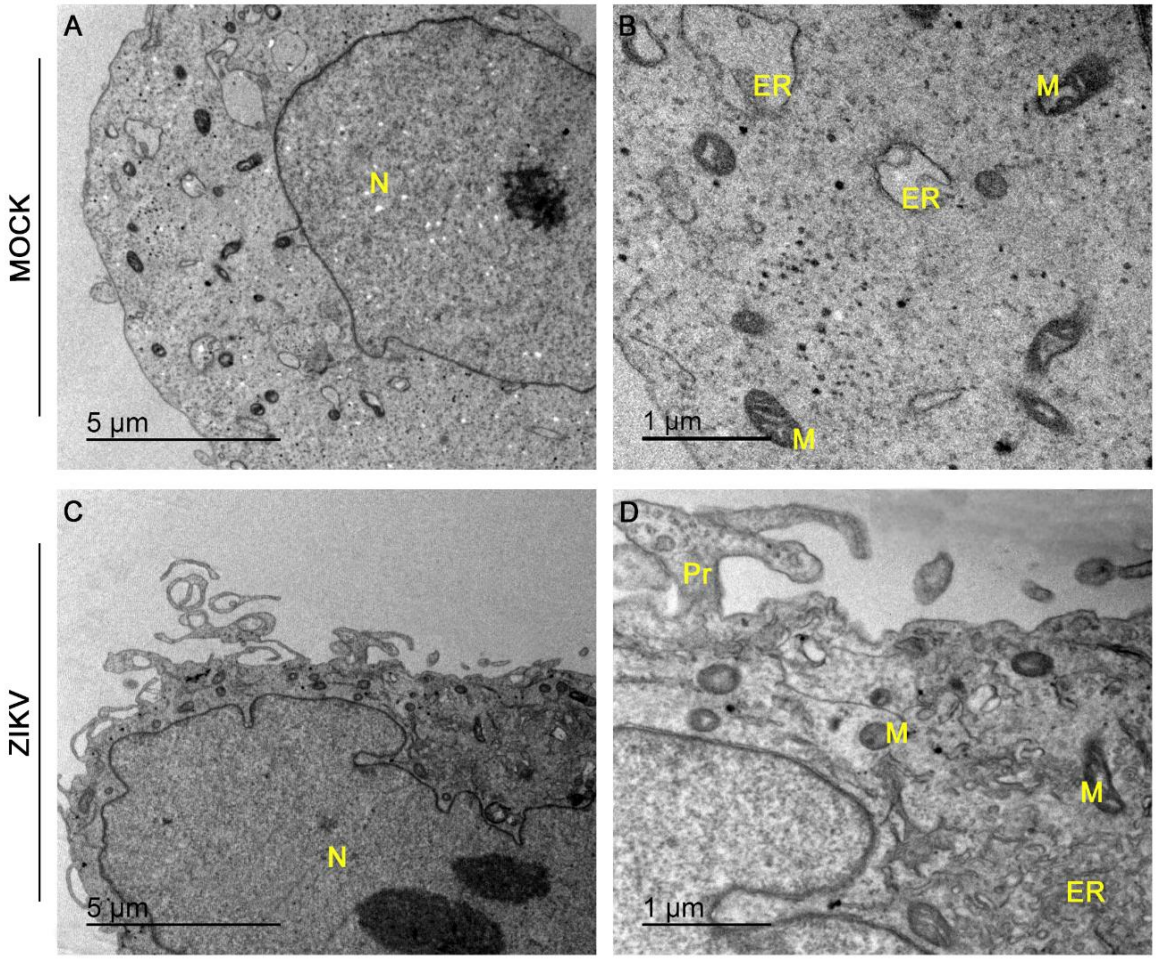

Figure 2. Ultramicrography of HTR8/SVneo cells infected with the Zika virus, visualized in Transmission Electron Microscope. A-B) HTR-8/Svneo cells incubated with the Mock. No changes in organelles and cell cytoplasm were observed. C-D) HTR-8/SVneo cells infected with the ZIKV. The cells presented alterations such as increased cell prolongations (Pr) and secretion of vesicles, mitochondria (M) with loss of mitochondrial ridges and abundant endoplasmic reticulum (ER) with less dilated cisterns. Nucleus (N).

To evaluate the biochemical alterations related to oxidative stress in ZIKV infected HTR-8/SVneo cells, we performed an experiment to detect the activity of some enzymes, such as SOD, which presents a significant increase in its activity compared to control (1.29±0.41 and 0.21±0.11 U/mg of protein, respectively) (Figure 3A). On the other hand, the CAT enzyme activity was decreased compared to the control (5.1±3.8 and 33.88±30.0 U/mg of protein, respectively) (Figure 3B). We also analyzed some products of stress like MDA and NO. The MDA was significant increased compared to control (0.01±0.001 and 0.003±0.002 nmol/mg of protein, respectively), as well as the nitric oxide (95.68±52.01 and 48.79±9.18 μmol/ml, respectively) (Figure 3C-D).

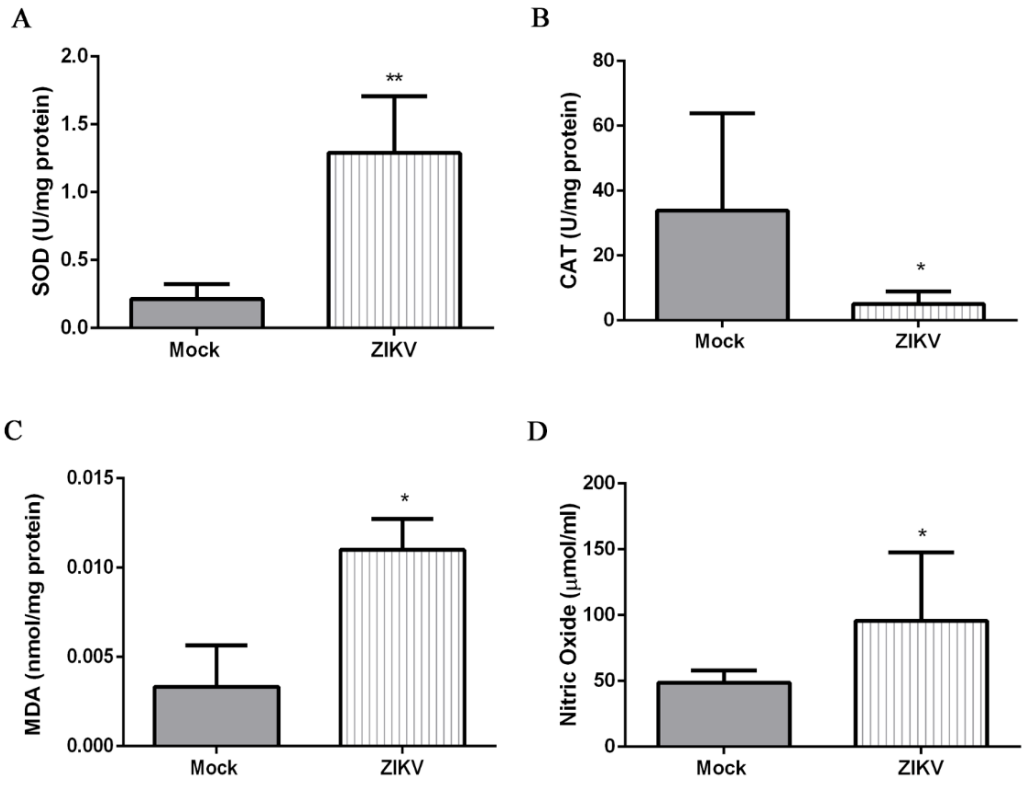

Figure 3: Analysis of biochemical changes related to oxidative stress in HTR8/SVneo cells infected with the Zika virus. A) Significant increase in SOD antioxidant enzyme activity compared to control, B) significant decrease in CAT enzyme activity compared to the control, C) significant increase in MDA product compared to control, and D) significant increase in nitric oxide compared to the control.

Discussion

As the Zika virus has a large tropism for neuronal cells in fetuses, many *in vitro* studies have focused on the consequences that the virus brings to these cells [13–15]. In this work, we evaluated the consequences and damage that the virus brings to HTR-8/SVneo placental line, a cell type by which the virus also has tropism and affects before it even reaches the fetus. We standardized an infection protocol with the best MOI of virus, detecting

the amount of positive cells by immunofluorescence and flow cytometry, already evaluating the permissiveness of the HTR-8/SVneo, with the detection of NS1 protein, which is only present after viral replication. Other studies had already shown viral replication in this lineage, but using the detection of the amount of viral RNA [12].

Using the efficient condition to infect the trophoblastic cells, we observed the differences in ultrastructure of trophoblastic organelles after infection. The ZIKV seems activates the cells, exacerbating a process related to the abundant and evident endoplasmic reticulum, the formation of prolongations and release of microvesicles. Microvesicles are usually released by trophoblastic cells, rich in proteins that function in signaling and immunoregulation [16]. Another study noted that the inflammatory or stress stimulus can lead to increased secretion of these vesicles by HTR-8/SVneo [17]. We also observed loss in mitochondrial crest, a characteristic of irreversible damage to these organelles. These mitochondrial changes have already been observed by our group in the placental cells of a patient infected with ZIKV [18]. Besides, similar mitochondrial damage has been identified in other cell types in infection with another flavivirus, such as dengue [19,20].

With the mitochondrial injury observed in the ultrastructure and knowing that other flaviviruses cause oxidative stress to infected cells, we investigated the oxidative stress caused by ZIKV infection in trophoblastic cells [7]. The oxidative stress results from an imbalance between ROS, prooxidant, and antioxidant chemical species that leads to cellular oxidative damage and the mitochondria is the main site of ROS production. Endogenous enzymes are part of antioxidant systems within cells, and in our study, we evaluated SOD and CAT activity. SOD is responsible for converting the superoxide anions (produced by an electron leak from electron transport chains) into hydrogen peroxide and its activity was significantly increased in ZIKV infected cells [7,21]. This was an expected result, as consequence of mitochondrial damage and overproduction of superoxide anions. CAT activity is often the opposite of SOD activity, and it was no different in our analyzes. The CAT is the enzyme that acts in sequence, converting the hydrogen peroxide into oxygen and water. There was a decrease in its activity, probably due to its availability within the cell, which is lower than SOD and because of rapid consumption and exhaustion of these enzymes storage[22–24].

Another evidence of the damage caused to the placental cells by infection was the accumulation of MDA, a product of fatty acid peroxidation [25]. Despite the damage that MDA causes was not fully elucidated, it is known that this molecule is able to interact with nucleic acid bases to form several different adducts and causing mutations [25,26]. Nitric oxide is a soluble gas widely known for its role in circulation control and vasodilation modulation, as well as in synapses, or inflammation [27–29]. Its ability to be beneficial or potentially toxic because of tissue concentration or purification makes it a complex and antagonistic molecule. The high concentration of this reactive nitrogen species is potentially toxic, since its reaction with the superoxide anions generates peroxynitrite, which is highly reactive and can generate serious damage inside the cell, as well as in the external environment, since they are liposoluble [29]. Studies investigating oxidative stress due to ZIKV are still scarce and more studies should be carried out for further insight into the consequences of this virus, especially on placental cells [7].

Conclusion

The ultrastructural analysis of HTR8/SVneo cells infected with the ZIKV presented morphological changes compared to cells incubated with MOCK. Along with this, biochemical alterations profile of the infected cells were observed, indicating oxidative stress. These results will help to the better understand about the pathogenesis of the disease, elucidating the consequences of infection in placental cells.

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3 **DISCUSSÃO**

3.1 **Estudos em placentas de pacientes infectadas por ZIKV e órgãos de natimorto**

A transmissão vertical é uma das maiores preocupações na infecção com ZIKV. As alterações congênitas nunca haviam antes sido diretamente associadas a infecções por flavivírus, e a compreensão que tivemos no surto de Zika de 2015-2017 sobre as infecções congênitas baseou-se apenas em estudos retrospectivos de outras doenças. Devido ao pouco conhecimento a respeito da infecção por ZIKV na placenta, o estudo desse órgão nas mulheres grávidas infectadas, assim como o de modelos *in vitro* podem fornecer informações importantes. Estes podem ajudar a elucidar como ocorre a transmissão, quais as consequências para a placenta e para o feto, pois afinal há um prospecto epidemiológico de possíveis novos surtos dessa doença (197,198).

Não são muitos os vírus capazes de romper a barreira placentária e infectar o feto, ademais, há a necessidade urgente de compreender os mecanismos pelos quais isto ocorre, especialmente na infecção por ZIKV. Os vírus que chegam ao feto incluem o vírus varicela zoster (VZV), parvovírus B19 (PVB19), citomegalovírus humano (CMV), hepatite E 1 (HEV-1) e o ZIKV. Entretanto, existem outros vírus que podem infectar os recém-nascidos no momento do parto, incluindo o herpes simples 1 e 2 (VHS-1 e 2), o vírus da imunodeficiência humana (HIV), assim como os vírus da hepatite A-C (199). No primeiro trabalho desta tese, investigamos o caso de uma paciente soropositiva para o HIV, que também foi infectada pelo ZIKV durante sua gestação. Os danos característicos da SCZ foram observados nos exames realizados no segundo trimestre de gestação, sugerindo que ZIKV havia chegado ao feto, mas como confirmados pelos exames pós-natais, o bebê não foi infectado pelo HIV.

As avaliações clínicas do bebê após o nascimento mostraram alterações no crânio e no cérebro compatíveis com a SCZ, como a atrofia cerebral, ventriculomegalia, hidrocefalia, assimetria do globo ocular e múltiplas calcificações intracranianas (22,137,200,201). O acometimento do feto sugere que o ZIKV possui tropismo pela placenta, tendo um comportamento diferente do HIV, que só entraria em contato com o feto no caso de uma lesão placentária que favorecesse o contato deste diretamente com o sangue materno. A confirmação de que o ZIKV não só foi capaz de entrar nas células da placenta, mas também de se replicar, foi possível com a detecção da proteína E, constituinte da partícula viral e que

medeia a entrada do vírus nas células, e da proteína NS1, que é encontrada apenas na replicação viral (42). Adicionalmente, nas células do citotrofoblasto do caso em questão foram encontrados na análise ultraestrutural, aglomerados de partículas virais com tamanho compatível com o ZIKV, de cerca de 25 nm e nenhuma partícula compatível com HIV, que tem aproximadamente 120 nm (202,203).

Em contrapartida, o antígeno da proteína p24 do HIV não foi detectado na placenta, a transmissão vertical deste vírus pode ter sido evitada pelo uso de medicamentos anti-retrovirais, ou até mesmo pela diminuição da carga viral junto a outra infecção, já que dados publicados indicam que a infecção por outro flavivírus, como a dengue, está associada a uma diminuição transitória da carga viral de HIV, e não é observada nenhuma progressão grave durante a coinfecção (204,205). Um estudo que correlaciona as características dos bebês recém-nascidos de gestações de mulheres infectadas por HIV ou HIV-negativas descreveram que a circunferência da cabeça ao nascimento era semelhante nos dois grupos e na análise das placentas destas pacientes, não havia lesões específicas nas placentas infectadas (204).

Dois trabalhos também relataram coinfecção HIV/ZIKV em pacientes no Rio de Janeiro, um homem que manifestou apenas sintomas leves e boa recuperação, e uma mulher grávida, que sofreu aborto com 20 semanas de gestação e apresentou algumas alterações histopatológicas na placenta (206,207). Um outro estudo descreveu o perfil clínico de cinco pacientes HIV soropositivos, todos com boa resposta ao tratamento anti-retroviral, que foram infectados por ZIKV. Dois deles apresentaram sintomas da doença, mas com quadro leve, enquanto os outros três tiveram um perfil mais grave, com diminuição da força muscular, limitação da marcha, comprometimento do nervo facial, relaxamento de esfíncteres, arreflexia, sendo a desmielinização identificada pela electromiografia, caracterizando o quadro de Síndrome de Guillain-Barré (208).

Os sintomas desses pacientes foram similares aos observados no caso da gestante infectada durante o surto de Zika em 2016, que investigamos no segundo trabalho desta tese, no qual ela apresentou os sintomas clássicos de febre da Zika, como erupção cutânea, prurido e vômito, e após um mês evoluiu para o quadro de SGB com sintomas que incluíam tetraparesia, parestesia, arreflexia, disautonomia e sinais de insuficiência respiratória, confirmados por eletroneuromiografia. Estes sintomas são os mais comumente descritos na SGB associada à infecção por ZIKV, que possui um quadro de morbidade mais acentuada, provavelmente pelo fato deste vírus ter um maior tropismo pelo sistema nervoso (157,209). Outro trabalho, com casos de pacientes que apresentaram sintomas semelhantes aos

encontrados no caso investigado pelo nosso grupo, observaram que a viremia dos pacientes persistiu por mais tempo que o normal (210).

Um estudo que discute as possíveis formas de infecção das células nervosas levando à SGB, cita que o fenômeno do *antibody-dependent enhancement* (ADE) poderia ser um dos facilitadores deste processo, cujos anticorpos produzidos numa infecção primária por outros flavivírus, como dengue, formam complexos com ZIKV sem neutralizá-los, e estes complexos vírus-anticorpos são endocitados por células que possuem receptores Fc, aumentando assim a replicação viral (211). Este fenômeno já foi investigado e teve resultados confirmando danos diretos ao tecido placentário, com aumento da replicação de ZIKV (212).

Com base nas informações imunológicas decorrentes da doença e na entrada de ZIKV nas células, Muñoz et al., desenvolveram uma outra teoria sobre a patogênese de ZIKV/SGB. O dano neuronal poderia ocorrer através da ativação do sistema complemento e o ZIKV agiria diretamente nas células (a bainha de mielina ficaria intacta), tomando as características desenvolvidas em AMAN, enquanto a desmielinização que é atributo do AIDP seria induzida pela inflamação mediada por células, complemento e ativação de macrófagos (211). Todos os subtipos de SGB já foram associados à infecção pelo ZIKV, mas as proporções destes subtipos variam entre os estudos e as regiões (213).

Embora as investigações mostrem que os homens são mais suscetíveis à SGB, no caso da síndrome em associação com a infecção por ZIKV, a preocupação com as pacientes grávidas é muito relevante (214). Os estudos que descrevem os casos de SGB em gravidez são raros e pouco se sabe sobre as alterações imunológicas e outros fatores de risco que podem tornar as mulheres grávidas mais propensas aos distúrbios neurológicos. Até o momento, nosso trabalho foi o único que descreveu o caso de uma paciente grávida infectada por ZIKV com SGB. O desfecho da paciente grávida foi de recuperação da SGB após 1 ano de acompanhamento e tratamento, mas a gestação de 15 semanas não foi sustentada, gerando um aborto espontâneo retido. Apesar de a relação entre a febre da Zika e a SGB na gestação ainda carecer de maiores investigações, a descrição da influência viral nas malformações congênitas tornou-se menos enigmática e pode-se inferir que o aborto é uma das possíveis consequências da infecção por ZIKV (151,215,216). Após a curetagem, alguns órgãos do natimorto foram coletados para análise.

Sendo assim, investigamos os aspectos histopatológicos e detectamos os antígenos virais no cérebro, pulmão, pele, rins e fígado do natimorto, comparados com órgaos de um natimorto controle, com a mesma idade de desenvolvimento, proveniente de um aborto espontâneo de causas não infecciosas. Em todos os órgãos avaliados, foram observados

diferentes tipos de lesões, desorganização tecidual e os antígenos virais foram detectados. Os órgãos foram positivos na análise por imunohistoquímica tanto para a proteína estrutural E, quanto para a proteína NS1, que evidencia a replicação do vírus nesses órgãos. Outros trabalhos também mostraram a presença e replicação de ZIKV por imunohistoquímica ou por análises de RT-PCR em tecidos fetais e órgãos de natimortos ou bebês que foram a óbito em poucas horas ou dias após o parto (150–152).

O cérebro é um órgão pelo qual o ZIKV possui tropismo e um dos mais afetados na infecção transplacentária (97,147,217,218). A análise histopatológica do tecido cerebral do natimorto de 15 semanas investigado no presente estudo, demonstrou uma grave degeneração das fibras nervosas e desorganização das camadas do córtex cerebral. A morte celular das fibras nervosas também foi observada no tecido cerebral avaliado em outros casos (152,215). Entretanto, outros danos como edema, congestão vascular, calcificações e infiltrado linfocitário já foram observados nos tecidos cerebrais de bebês que chegaram à termo (152,219,220). As células positivas na imunomarcação para os antígenos virais foram os neurônios e as células da microglia, as mesmas relatadas em outro estudo com cérebros de natimortos (215).

No caso em questão, foram observadas áreas com desorganização da arquitetura dos bronquíolos, associada à áreas focais da membrana hialina nos pulmões analisados. Outras alterações incluíram regiões de espessamento septal, aumento da celularidade, necrose do epitélio respiratório e a presença de infiltrado de células mononucleares. O infiltrado inflamatório pulmonar também foi relatado no estudo conduzido por Martines et al., em bebês que nasceram com a SCZ e faleceram após 2 meses de vida, mas não houve imunomarcação positiva para ZIKV nos pulmões (215). No nosso trabalho, as proteínas E e NS1 foram detectadas nos macrófagos alveolares.

A pele do feto infectado apresentou áreas difusas de edema associadas a infiltrado linfocitário perivascular na região da derme, com células positivas para ambos antígenos virais testados nas células mononucleares. Não há até o momento outro trabalho que relate os achados na pele de um natimorto, feto ou bebê infectado por ZIKV. Esse é um importante órgão a ser melhor investigado na infecção transplacentária, visto que a pele é possivelmente a primeira barreira pela qual o vírus teria que passar para chegar aos outros órgãos, já que há a presença de partículas virais no líquido amniótico (221).

Nos rins, foram observadas algumas áreas de isquemia dos glomérulos, desorganização da morfologia e degeneração dos túbulos. Além disso, encontramos focos de infiltrado mononuclear e as células positivas na imunomarcação para os antígenos virais

foram os macrófagos. Corroborando nossos achados, Azevedo et al. encontraram túbulos com autólise, e outros danos como a congestão vascular e imaturidade dos glomérulos na histopatologia dos rins dos casos analisados (152).

O fígado do natimorto apresentou uma grave perda da morfologia do parênquima e desorganização da vascularização. As lesões mais proeminentes foram as degenerações celulares e na matriz extracelular, associadas ao aumento do número de células de Kupffer. A autólise e o infiltrado celular foram relatados em outros casos, assim como esteatose, congestão vascular e edema. Alé disso, as células positivas para ZIKV em um dos fígados avaliado por Azevedo et al. foram as mesmas em que detectamos as proteínas E e NS1 no nosso trabalho, os hepatócitos e as células de Kupffer (152).

Já foi descrito o acometimento de órgãos viscerais e do cérebro em fetos e adultos em casos fatais de outros flavivírus, como o da dengue, (222–226). Um estudo sobre os órgãos de um caso de natimorto, relatou cérebro com grande infiltrado inflamatório; danos no fígado, como infiltrado monuclear, necrose dos hepatócitos, esteatose e hiperplasia de células de Kupffer; além de alterações pulmonares como hialinose, infiltrado monuclear e espessamento alveolar. Em todos esses órgãos foi detectada a proteína NS3 do vírus da dengue e houve um aumento nas células e citocinas relacionadas à inflamação, como células $CD68^{+}$, T $CD8^{+}$ e citocinas RANTES, MCP-1, IFN-ɣ e TNF-α (222). Alterações semelhantes já foram observadas nesses mesmos órgãos em casos fatais de adultos com dengue. Análises histopatológicas revelaram, em diferentes casos, áreas com edema e hemorragia, esteatose e necrose no fígado; necrose tubular renal;espessamento dos septos alveolares e formação de membrana hialina nos pulmões estudados. Os antígenos virais são encontrados nesses órgãos e há neles um ambiente pró-inflamatório estabelecido (223–226).

Nos quatro primeiros trabalhos apresentados nesta tese que investigaram as placentas de pacientes infectadas por ZIKV durante a gestação, realizamos uma minuciosa análise da histopatologia desse órgão. Os danos observados na decídua foram áreas grandes e difusas de necrose fibrinóide ou depósitos de fibrina, calcificação, edema, espessamento endotelial fibroso, degeneração celular, congestão vascular, áreas focais de infiltrado de células mononucleares e infiltrados inflamatórios perivasculares (deciduíte). A necrose fibrinóide é uma vasculopatia decidual, caracterizada pelo processo de necrose que ocorre nas paredes dos vasos e geram áreas impregnadas de fibrina, que aparecem vermelhas, róseas ou manchadas nas áreas histológicas afetadas coradas em H.E., sendo um dano característico na hipertensão (227). A calcificação é caracterizada pela deposição de cálcio na placenta, o que ocorre de forma natural no amadurecimento do órgão, sendo muito comum na placenta à termo. A

presença de calcificação placentária pré-termo é um preditor de mau fluxo uteroplacentário, o que pode levar a danos graves à manutenção da gravidez e ao feto (228,229). O espessamento endotetial também pode acontecer decorrente de hipertensão e é característico de disfunção endotelial decorrente de inflamação e estresse oxidativo, no qual esses fatores levam a proliferação celular na parede do vaso, seja das células endoteliais, musculares e dos fibroblastos, que acabam por aumentar a deposição de colágeno ao redor dos vasos (230). O edema, o infiltrado inflamatório e a congestão vascular ocorrem devido ao processo inflamatório e ao aumento da permeabilidade vascular, no qual o organismo entra em um processo de defesa contra o patógeno, liberando o exsudato e elevando a migração de células imunes do sangue para o local da lesão (231–233).

A principal alteração observada na porção fetal das placentas foi a imaturidade das vilosidades coriônicas, mas também observamos alterações inflamatórias aguda e crônica (infiltrado linfocitário), hiperplasia de células de Hofbauer, hipoplasia das vilosidades, áreas focais de hemorragia, e da mesma forma que houve nas decíduas, as vilosidades também apresentaram calcificação, edema, depósitos de fibrina, espessamento endotelial fibroso e congestão vascular. Além disso, poucos casos mostraram evidências de lesões isquêmicas como infarto. Outros achados foram a intervilosite, com presença de células imunes na região intervilosa e a congestão desse espaço interviloso. A maturação vilosa tardia é definida como a vascularização reduzida das vilosidades coriônicas, que gera uma vilosidade de aspecto mais "frouxo", com mais espaços pouco corados no H. E., formando vilosidades bolhosas. Ainda são poucos os trabalhos que falam sobre a imaturidade das vilosidades, mas sabe-se que principalmente no terceito trimestre da gravidez, pode estar associada a um risco aumentado de natimortos (234–236). A hipoplasia das vilosidades é caracterizada por árvores vilosas distais pouco desenvolvidas, com as ramificações alongadas, gerando grandes espaços intervilosos (235).

A principal alteração observada nas placentas dos casos estudados foi o atraso na maturação das vilosidades, também encontrada em estudos de placentas humanas infectadas por ZIKV, realizados por outros grupos (131,237–239). Entretanto, uma outra alteração de grande relevância é a hiperplasia das células de Hofbauer. Esse aumento nos macrófagos residentes vilosos corresponde ao que foi verificado em outros trabalhos e parece ter um papel importante na infecção por ZIKV, visto que esta é uma célula suscetível e permissiva a infecção (131,237,239). Além destas, as outras alterações notadas nos casos aqui investigados já foram observadas em outros trabalhos que avaliaram placentas infectadas por ZIKV (125,131,163,237,240). No entanto, as alterações placentárias encontradas na infecção pelo

ZIKV não são patognomônicas e podem ter características particulares em diferentes pacientes.

Além das modificações histopatológicas, observamos quais as células das placentas analisadas foram suscetíveis a infecção e se estas eram capazes de replicar o vírus, ou seja, se eram também permissivas. As proteínas flavivirais E e NS1, assim como a fita negativa do RNA, foram detectados em muitas células da placenta, nas decíduas e nas vilosidades analisadas, incluindo as células trofoblásticas. Podemos citar as células deciduais e endoteliais, citotrofoblastos, sinciciotrofoblastos, células mesenquimais e de Hofbauer, mastócitos e células imunes circulantes como células positivas para ZIKV nas nossas investigações. Esses resultados comprovam, juntamente com outros estudos, que as células placentárias são suscetíveis, já que encontramos a proteína E, que faz parte do envelope viral nas células. Além disso, são também permissivas à infecção pelo ZIKV, comprovado pela detecção da proteína não estrutural NS1, que só é encontrada após a replicação do vírus e da fita negativa do RNA, transcrito como molde para novos RNAs positivos. Tanto as imunomarcações quanto a detecção da fita negativa do RNA ocorrreram na região citoplasmática das células, o que condiz com o perfil normalmente encontrado na detecção destes antígenos (38,41,42). Estes achados corroboram a teoria de que o vírus pode atingir o feto por infecção célula a célula, rompendo a barreira placentária (75,163,164).

Os antígenos de ZIKV já foram encontrados nas mesmas células placentárias em outros estudos que utilizaram imunohistoquímica, assim como o RNA viral extraído do tecido placentário detectado por RT-PCR (163,215,238,241). Ademais, alguns estudos *in vitro* têm mostrado que linhagens de células trofoblásticas placentárias, explantes e células imunes são suscetíveis a infecção pelo vírus e que apresentam receptores capazes de se ligar ao vírus e mediar a entrada celular por endocitose (122,124,242,243).

Além das lesões observadas na histopatologia da placenta, observamos estas células em seus aspectos ultraestruturais. Nas células do tecido placentário do primeiro caso avaliado, notamos que os núcleos do sinciciotrofoblasto apresentaram cromatina dispersa agregada à membrana nuclear, e citoplasma rarefeito com ausência de organelas, comparados ao controle. Essa alteração nuclear sugere um possível dano ao DNA dessas células, que foi confirmado pela intensidade de expressão da proteína *proliferative nuclear cell antigen* (PCNA), uma proteína envolvida na replicação e no reparo do DNA, indicando uma tentativa das células de reparar o DNA lesionado (244). Alguns estudos mostraram que a ligação do PCNA para reparar outras proteínas é direta e que a inibição da sua expressão prejudica o mecanismo de reparo do DNA (245,246). O aspecto do sinciciotrofoblasto nas outras

placentas infectadas avaliadas foi também bastante modificado, com presença de grandes vesículas e corpos apoptóticos no citoplasma, ausência dos seus prolongamentos de membrana e da secreção de microvesículas, caracterizando uma alteração morfológica e provavelmente funcional nestas células, que têm como característica sintetizar e secretar grandes quantidades de hormônios esteróides e peptídeos (247).

Adicionalmente, os citotrofoblastos analisados nas diferentes amostras apresentaram mitocôndrias menores, tumefeitas, com menos cristas mitocondriais, ruptura da membrana mitocondrial e o retículo endoplasmático exibiu cisternas mais dilatadas. A análise ultraestrutural dos tecidos placentários permitiram também a identificação em diferemtes pacientes de aglomerados com partículas virais, posicionados adjacentes ao retículo endoplasmático dos citotrofoblastos, ponto preferencial de replicação do vírus (248,249). Estas partículas tinham aproximadamente 25 nm de diâmetro, que são as dimensões do ZIKV (203).

É importante notar que o vírus continuou se replicando nas pacientes em todos os casos, mesmo meses após a infecção aguda de febre da Zika. Foram detectados diferentes antígenos virais em todas as placentas das pacientes analisadas por imunohistoquímica, além de algumas apresentarem a presença de partículas nas células placentárias por microscopia eletrônica, e em dois casos houve detecção de RNA viral por RT-PCR no soro. Todos os materiais analisados foram coletados no momento do parto, como evidências definitivas de uma infecção viral persistente nas pacientes, principalmente na placenta.

Além das alterações nas células dos tecidos placentários, pela análise ultraestrutural foi possível observar que nas amostras das pacientes infectadas não havia fibras de colágeno na maioria dos tecidos analisados, enquanto estas fibras eram sempre presentes e facilmente encontrados nas amostras controle. Esses achados corroboram com a análise que fizemos por microscopia ótica nessas placentas, coradas por *Periodic acid-reactive Schiff* (PAS) ou Picro Sírius Red, na qual observamos uma diminuição significativa de glicoproteínas e colágeno, indicando que o ZIKV leva a degradação da matriz extracelular. A matriz extracelular é fundamental para a manutenção da homeostase placentária, fornecendo valiosas percepções sobre a situação biológica do órgão. A matriz placentária é rica em glicoproteínas e colágeno, principalmente os colágenos I e IV (250). Um dos fatores que provavelmente contribuíram para a degradação observada nos casos analisados foi o aumento significativo das metaloproteínases 2 e 9, também chamadas de gelatinases A e B, respectivamente. A MMP-2 é responsável pela clivagem de glicosaminoglicanos como o condroitin-sulfato e dos colágenos I, II, III, IV e V, enquanto a MMP-9 cliva os colágenos IV, V e XI e fibronectina

(251,252). O aumento dessas enzimas pode ter sido responsável pelo aspecto de tecido mais frouxo e de vilosidades bolhosas, característico da imaturidade vilosa que observamos nas placentas infectadas. Essas metaloproteinases têm sido mais investigadas devido ao seu papel na implantação e relação ao remodelameno tecidual extensivo, pois já foi observado o seu aumento em placentas de mulheres que tiveram parto prematuro (253–255). Além das próprias células placentárias sintetizarem as MMPs contitutivas, estas enzimas têm a produção exarcebada no contexto inflamatório, já que monócitos, macrófagos e células T citotóxicas também são capazes de produzi-las após estimuladas por citocinas (256,257).

Com os achados histológicos de infiltrado inflamatório, hiperplasia de células de Hofbauer, e a imunomarcação das proteína E e NS1 nesssas células, realizamos algumas investigações a fim de avaliar o contexto inflamatório nas placentas das pacientes infectadas por ZIKV. Observamos inicialmente que as células imunes das placentas dos dois primeiros casos analisados eram capazes de replicar o vírus, pela co-localização da proteína CD11b, uma integrina usada como marcador para leucócitos e especialmente células T $CD8^+$ efetoras, macrófagos e granulócitos, com a proteína NS1 de ZIKV (258,259).

Em relação às imunomarcações e quantificações de células $CD68^+$ realizadas nas placentas das pacientes do segundo caso, que apresentaram SGB ou das dez pacientes positivas para ZIKV analisadas no terceiro trabalho da tese, observamos um aumento significativo da população de macrófagos local, comparados aos controles e detectamos por fluorescência que os macrófagos $CD163^+$ (ativados) foram capazes de replicar o vírus. As células de Hofbauer e os macrófagos deciduais são residentes na placenta e possuem um papel regulador na gravidez, além de serem capazes de manter um ambiente homeostático essencial ao desenvolvimento fetal (260). A infecção dos macrófagos deciduais e das células Hofbauer durante o período pré-natal não apenas reflete a falha crítica na proteção materno-fetal, mas também destaca um caminho potencial para a transmissão vertical do ZIKV. De fato, vários grupos de pesquisa demonstraram tanto histologicamente ou por culturas e explantes isolados que esses macrófagos placentários são altamente permissivos à replicação do ZIKV (131,213,237,239,243,261). Essa observação corresponde aos achados de Rosenberg et al., que em um estudo histológico de um caso de Zika também detectaram proliferação e hiperplasia dessas células placentárias residentes (239). Além disso, o aumento de células $CD163^+$ na placenta já foi sugerido com um dos fatores associados ao parto precoce e ao dano fetal induzido pelo ZIKV (165).

Outro fato importante que chamou atenção foi o aumento expressivo de células T $CD8^+$ nessas mesmas placentas. As células T $CD8^+$ (chamadas também de citotóxicas) são

capazes de matar diretamente as células infectadas, além de produzir citocinas efetoras, o que as tornam essenciais para o controle de infecções virais (262). Como amplamente investigado, a imunidade celular mediada por estes linfócitos é crítica para a defesa do hospedeiro contra a infecção pelo ZIKV (213,263–267). O aumento de linfócitos T $CD8^+$ foi observado em primatas não humanos após a diminuição da viremia, o que sugere um papel protetor das células T $CD8^+$ no controle da replicação do ZIKV (268). Ademais, estas células são capazes de gerar populações de memória de longa duração, a fim de responder rapidamente à reinfecção e fornecer maior proteção (263). Esse resultado corrobora que há a ativação da resposta imune no tecido em consequência da infecção, sendo uma resposta majoritariamente citotóxica, como esperado para a infecção viral, já que nessas placentas, a detecção de células T $CD4^+$ foi baixa e inconclusiva. Nos casos analisados em nosso estudo, a maioria das pacientes foi IgG negativa ou não reativa à dengue, o que poderia torná-las menos protegidas, entretanto o caso 7 do terceiro trabalho foi IgG positivo para dengue, e mesmo tendo possíveis células T $CD8^+$ de resposta cruzada, ainda apresentou um aumento na migração destes linfócitos para a placenta.

O aumento de macrófagos e linfócitos T $CD8^+$ caracterizam um ambiente inflamatório crônico na placenta, com lesões como deciduíte e vilosite observadas em todos os casos (269). Sabe-se agora que a ativação imune materna (MIA) é dinâmica e a imunidade é essencial para o desenvolvimento da gravidez, desde a implantação até o parto, e é muito eficaz na prevenção de infecções virais (270–272). No entanto, o ZIKV estabelece uma infecção placentária, ignorando a MIA e promovendo a inflamação como uma resposta relevante. Esse ambiente é capaz de se estabelecer através da liberação de citocinas pró-inflamatórias, como TNF-α e IFN-ɣ, citocinas bastante exacerbadas nos casos analisados, que exercem quimiotaxia e ativam essas células, aumentando também a expressão do MHC-1 e induzindo ação ainda mais intensa dos linfócitos citotóxicos (273,274). O TNF-α é uma citocina produzida por diferentes células no ambiente inflamatório, como os monócitos, macrófagos e linfócitos para uma defesa contra patógenos na resolução da inflamação, sendo responsável pelo recrutamento de outras células imunes, promovendo o remodelamento e a destruição das células infectadas (273). O IFN-ɣ (ou INF do tipo 2) é uma citocina produzida majoritariamente por células T efetoras na resposta imune adaptativa mediando a resposta antiviral com a ativação de macrófagos, recrutamento de outros linfócitos para o local da lesão e regulando a proliferação e apoptose celular local (274).

Além da resposta mediada por células, das seis amostras de soros de pacientes que analisamos por PRNT, cinco foram positivas para a produção de anticorpos neutralizantes,

sugerindo que há nessas pacientes uma resposta imune humoral capaz de interferir na capacidade de infecção do ZIKV (275). A produção desses anticorpos têm sido bastante discutida e é paradoxal, pois alguns pesquisadores entendem que essa produção pode aumentar a reatividade cruzada ou levar a proteção no caso de uma nova infecção por outros flavivírus, dependendo do tempo que separa as duas infecções e da frequência da exposição. Entretanto, para uma nova exposição ao ZIKV, esses anticorpos seriam bastante eficientes junto a resposta celular e capazes de evitar a doença (276).

O *Vascular endothelial growth factor receptor 2* (VEGFR-2) é um receptor do tipo tirosina quinase, conhecido principalmente por ser expresso em células endoteliais. Este receptor responde à ligação ao fator de crescimento endotelial vascular (VEGF), que inicia uma cascata de fosforilação, levando à transdução de sinais que regulam a transcrição gênica no núcleo, resultando em maior proliferação e migração endotelial (277). A quimiocina RANTES/CCL5 desempenha um papel nas respostas imunes a infecções virais, sendo um regulador para a ativação das células T, expresso por estes linfócitos e também em células endoteliais. O mediador RANTES atua atraindo muitos tipos de células, como monócitos, células NK, células T de memória, eosinófilos e células dendríticas, mas possui relação também com a disfunção endotelial e angiogênese (278–282). Os níveis de receptor VEGFR-2 e do mediador RANTES também foram significativamente elevados nos tecidos estudados, o que leva a crer que as placentas infectadas estavam sofrendo alterações vasculares e endoteliais relacionadas ao aumento da permeabilidade vascular, podendo causar uma grande disfunção circulatória que resultou nas alterações observadas como o depósito de fibrina e a congestão vascular. O depósito de fibrina na placenta pode refletir em abortos espontâneos e partos prematuros, afetando diretamente o desenvolvimento do feto e da gravidez (283). Outros autores também propuseram que a inflamação placentária induzida pelo ZIKV, ou outros vírus, teria um impacto crítico no desenvolvimento normal do feto (89,270). A expressão do VEGFR já foi relacionada a outras patologias na placenta, assim como RANTES foi observado na infecção placentária por ZIKV (126,284,285). Essas alterações podem levar também ao edema tecidual, resultando na falha de distribuição de nutrientes e hormônios, prejudicando a manutenção da homeostase tecidual e da gravidez.

Nas análises do perfil de células imunes e citocinas do terceiro trabalho, não houve diferença estatística entre as amostras dos grupos de placentas de pacientes positivas para Zika que tiveram bebês sem microcefalia ($ZIKV^+$ MIC^-) e de pacientes positivas para Zika que tiveram bebês com microcefalia ($ZIKV^+$ MIC^+). Em algumas quantificações, houve uma diferença no valor de p (probabilidade de significância) em cada um desses grupos

separadamente em comparação ao grupo controle, entretanto, o grupo ZIKV^{+} MIC^{-} sempre apresentou menor significância do que o grupo ZIKV^{+} MIC^{+}. A única exceção foi a quantificação do IFN-ɣ, na qual não houve diferença entre o grupo ZIKV $^{+}$ MIC^{-} e o controle, mesmo que este também tenha aumentado. Ou seja, a produção de IFN-ɣ foi a citocina do processo inflamatório com maior potencial em ter alguma relação com os casos de microcefalia. Nossos resultados mostraram que há uma grande inflamação da placenta na infecção pelo ZIKV, mas se é determinante para o desenvolvimento fetal cerebral, é devido a uma diferença sutil. Por esse motivo, decidimos avaliar também a quantidade de BDNF da placenta, um fator que já foi descrito como determinante para o desenvolvimento do cérebro (286,287).

O BDNF é um fator neurotrófico, produzido no tecido placentário, que desempenha um papel importante na diferenciação e proliferação do citotrofoblasto (288,289). Além disso, essa neurotrofina promove crescimento e diferenciação neuronal no sistema nervoso central e periférico durante o desenvolvimento fetal (290,291). As placentas de pacientes infectadas pelo ZIKV, principalmente do grupo de neonatos que manifestou microcefalia, apresentaram uma diminuição na expressão do BDNF, o que pode ser sugerido como um dos marcadores do dano ao desenvolvimento cerebral fetal.

Com todos os estudos que realizamos no tecido placentário de diferentes pacientes infectadas por ZIKV, elucidamos que muitas células placentárias são suscetíveis e permissivas à infecção por ZIKV. Além disso, observamos que há um grande envolvimento de células imunes e citocinas pró-inflamatórias no tecido infectado, levando a alterações na ativação e recrutamento de células circulantes, além de alterações na matriz extracelular e na permeabilidade vascular. Além disso, o cenário inflamatório da placenta parece ter um limiar discreto para a contribuição na microcefalia fetal, no entanto, o BDNF pode ser um marcador promissor para a investigação de alteração cerebral. As descobertas deste trabalho acrescentam bastante à descrição atual da patogênese do ZIKV.

3.2 **Estudos com as linhagens de células humanas HMC-1 e HTR-8/SVneo**

Observamos nos estudos realizados nas placentas das pacientes infectadas por ZIKV um perfil inflamatório local bastante evidente. Além disso, com a grande epidemia de Zika no Brasil, observamos que os pacientes possuíam exantema e prurido muito intensos no pico da

viremia, sintomas estes que eram aliviados pela administração de antialérgicos (22,176,189). Com base nessas informações sobre os sintomas e seu tratamento, notamos que os mastócitos poderiam ter algum envolvimento na infecção. A partir destas informações, elaboramos uma hipótese para o desenvolvimento do quarto trabalho desta tese, a fim de avaliar se os mastócitos seriam células suscetíveis e permissivas a infecção e se poderiam estar presentes na placenta, facilitando o processo inflamatório e a transmissão vertical.

Os mastócitos são células residentes encontradas abundantemente no endométrio e na placenta, e têm papel de destaque nas reações imunológicas (94,95). Além disso, acredita-se que os mastócitos possam desempenhar múltiplos papéis distintos neste local, desde a implantação até migração trofoblástica e a angiogênese (96,292). Apesar disso, o seu papel na patogênese da Zika e a possível contribuição para a transmissão vertical ainda não havia sido estudado. Deste modo, analisamos duas amostras de tecidos placentários infectados por ZIKV, comparando com uma placenta controle. Com estas amostras, encontramos os mastócitos nas placentas de todas as pacientes e relatamos, pela primeira vez, a presença do vírus nestas células no tecido placentário apenas das mulheres soropositivas para Zika, através da detecção da proteína NS1 colocalizada com a proteína marcadora de mastócito c-Kit. A presença de NS1 comprova a permissividade destas células, já que a NS1 é encontrada apenas na replicação viral (41). Muitos estudos já descreveram a permissividade com a replicação do ZIKV em outras células da placenta e do sistema imunológico. Essas descrições são extensas em relação às células de Hofbauer e aos macrófagos deciduais (213,237,239,243,261). Entretanto, as implicações das infecções por ZIKV nos mastócitos da placenta também podem ter relevância na compreensão do processo inflamatório local.

Após os resultados *ex vivo*, realizamos uma série de experimentos *in vitro* usando a linhagem de mastócitos humanos HMC-1, como um sistema modelo para desvendar aspectos de suas interações e consequências na infecção por ZIKV. Primeiramente, observamos na análise por citometria de fluxo que as células HMC-1 são suscetíveis à infecção com entrada do vírus, bem como sua rápida replicação em 30 minutos. Avaliamos com esse experimento, qual a quantidade adequada de vírus e o tempo em que há maior número de células infectadas. A melhor condição foi a de 1 *Multiplicity of infection* (MOI), com incubação de 24 horas. Como anteriormente citado, a replicação foi inferida pela detecção da proteína NS1 do Zika (41). Sabe-se que os mastócitos têm os receptores necessários para mediar a entrada do ZIKV, como HSP70 e HSP90, que também medeiam a entrada de outros arbovírus como o da dengue, além de estarem envolvidos nos sinais de transdução da cascata de degranulação (181,293).

Uma das proteases mais abundantes presentes nos grânulos dos mastócitos e que é amplamente usada para avaliar a degranulação, é a β-hexosaminidase, uma enzima glicolítica que é liberada nos tecidos e desencadeia reações típicas nas respostas alérgicas e inflamatórias (294). Por isso, utilizamos a quantificação da β-hexosaminidase no sobrenadante das células em cultura como medida da degranulação de mastócitos após incubações com diferentes MOIs de ZIKV. Utilizamos também o composto sintético 48/80 como um controle positivo, já que ele é um degranulador padrão para liberação de β-hexosaminidase pelas células HMC-1 (295). Houve um aumento significativo na liberação de β-hexosaminidase pelas células HMC-1 após contato com o ZIKV, que só foi detectado em 30 minutos, o que nos leva a crer que a adsorção viral é um estímulo para a degranulação. O período de 30 minutos é consistente com os da adsorção e internalização de partículas de flavivírus, que ocorrem rapidamente em cerca de 13 a 15 minutos, conforme observado anteriormente em outro trabalho realizado com partículas do vírus da dengue (296). Com 1 MOI, os níveis de β-hexosaminidase foram mais próximos aos do controle positivo com 48/80. A degranulação, detectada pela liberação de β-hexosaminidase, já foi associada à adsorção e infecção pelo vírus da dengue em outro estudo (179). A clivagem de alguns substratos dessa enzima tem sido associada à diferenciação de células NK, e a alta atividade da β-hexosaminidase já foi observada em algumas disfunções placentárias como a degradação de trofoblastos e pré-eclâmpsia (294,297).

Além das enzimas liberadas durante a degranulação, os mastócitos são responsáveis pela produção e liberação de diferentes fatores, citocinas pró e anti-inflamatórias. Neste contexto, avaliamos a produção de TNF-α, IL-6 e IL-10 em diferentes momentos, desde a adsorção viral e infecção com 30 minutos até 6h e 24h após a infecção. No momento do contato inicial dos mastócitos com o mock ou o vírus, houve uma liberação dessas citocinas e VEGF, o que é esperado, já que os mastócitos possuem reservas internas que são liberadas em resposta a um estímulo. Como o sobrenadante das células Vero (mock) possui um rico secretado de proteínas, esse estímulo parece ter sido suficiente para a liberação em 30 minutos. No entanto, ao final de outros tempos de incubação, os controles apresentaram baixos níveis de secreção desses mediadores, diferentemente das células infectadas com ZIKV. Houve um aumento significativo nos níveis das citocinas após 6h, o que seria esperado para gerar um ambiente propício ao recrutamento e diferenciação de outras células imunes. O TNF-α é uma das citocinas características da inflamação, que é produzida para a defesa contra patógenos, orquestra o recrutamento de células imunes e promove a remodelamento dos tecidos (273). A citocina IL-6 possui papel crucial na inflamação e também leva ao

recrutamento e diferenciação dos próprios mastócitos, além de monócitos, células T $CD4^+$ e $CD8^+$, ativação de linfócitos B e estimula a produção de VEGF por fibroblastos. A expressão de IL-6 afeta os processos homeostáticos relacionados à lesão do tecido e à ativação de respostas relacionadas ao estresse (298–301). Apesar de ser uma citocina anti-inflamatória, a expressão de IL-10 aumentou nas células HMC-1 infectadas com ZIKV, o que corrobora o que foi observado em outro estudo, no soro de pacientes Zika positivo (302). Além disso, a IL-10 é uma citocina normalmente produzida por mastócitos ativados, que leva à ativação de outros mastócitos e está presente em respostas alérgicas (303,304). Nossos achados *in vitro* corroboram com o aumento de citocinas e o ambiente inflamatório que observamos na infecção placentária por ZIKV, com o aumento de TNF-α e do receptor VEGFR-2. Além disso, o TNF-α combinado com o VEGF já foram relacionados à disfunção placentária vascular, levando ao extravasamento de plasma e pré-eclâmpsia (305). Outro estudo mostrou que a estabilização dos mastócitos pode diminuir sua resposta e minimizar a gravidade da dengue, relacionada à liberação de VEGF e à permeabilidade vascular (306).

As mudanças ultraestruturais que ocorrem na infecção podem ser bastante esclarecedoras em relação aos efeitos que a célula sofre com o patógeno. Nas investigações em linhagens de células de mastócitos HMC-1 que submetemos a infecção por ZIKV, analisamos as alterações ultraestruturais após 30 minutos e 24h de infecção. As células incubadas com o ZIKV durante 30 min têm uma taxa de infecção baixa, entretanto, se encontram no momento ótimo de adsorção viral, desencadeando a degranulação. Pela ultraestrutura, observamos uma diminuição dos grânulos celulares, sem outras alterações importantes. Após 24 horas, os mastócitos infectados apresentaram várias alterações como a formação de numerosas vesículas, cisternas do retículo endoplasmático dilatadas, características de um alta produção proteica, além de mitocôndrias tumefeitas, rupturas nas membranas celulares e ausência de núcleo sugerindo que uma parte das células pode ter entrado em processo de morte celular (307,308). Em várias células foi detectada a presença de partículas virais que correspondem ao tamanho de uma partícula de ZIKV (203). Não há outro trabalho na literatura investigando a infecção de mastócitos por ZIKV e suas alterações em consequência à infecção.

Nossos dados são evidências de que os mastócitos são permissivos à infecção pelo ZIKV, que a infecção pode induzir degranulação em seu primeiro contato e levar a produção de citocinas e VEGF após algumas horas de infecção. Essa resposta dos mastócitos pode facilitar a instalação de um ambiente pró-inflamatório nos locais onde essas células são

encontradas, como a placenta. Além disso, o fato de serem permissivas à replicação do vírus na placenta humana sugere que essa célula pode contribuir para a transmissão vertical.

Além da linhagem de mastócitos, no quinto estudo desenvolvido nesta tese, utilizamos também para nossas análises a linhagem de trofoblastos humanos HTR-8/SVneo, um tipo de célula para o qual já observamos que o vírus apresenta tropismo e atinge antes mesmo de chegar ao feto. Devido a algumas limitações para trabalharmos com o tecido placentário, escolhemos estas células como modelo *in vitro* para investigarmos melhor os efeitos da infecção por ZIKV em células placentárias. As investigações envolvendo os principais mecanismos patogênicos, como o estresse oxidativo, é uma lacuna a ser preenchida em relação à infecção pelo ZIKV, principalmente nas células placentárias. Como o vírus da Zika tem um grande tropismo por células neuronais em fetos, muitos estudos *in vitro* focaram nas conseqüências que o vírus traz para essas células (60,64,147).

Inicialmente, padronizamos um protocolo de infecção com a melhor MOI de vírus, detectando a quantidade de células positivas por imunofluorescência e por citometria de fluxo, avaliando também a permissividade da HTR-8/SVneo, com a detecção da proteína NS1 de ZIKV. Desta forma, estabelecemos que 1 MOI em 24 horas de infecção foi o ideal para a realização dos outros experimentos, pois foi a condição em que houve maior número de células infectadas. Outro estudo já observou replicação de ZIKV nessa linhagem, mas usando apenas a detecção da quantidade de RNA viral (309).

Utilizando a condição mais eficiente para infectar as células trofoblásticas, observamos as diferenças na ultraestrutura das organelas celulares ao longo da infecção. Após 24 horas de infecção, as células apresentaram prolongamentos celulares, secreção de vesículas e retículo endoplasmático abundante. Essas alterações parecem estar exacerbando um processo já realizado pelas células trofoblásticas, que é a ampla produção de proteínas e sua secreção que funcionam como sinalização e imunoregulação (310). Outro estudo observou que o estímulo inflamatório ou de estresse pode levar ao aumento da secreção destas vesículas pela HTR-8/SVneo (311). Observamos também perda nas cristas mitocondriais, característica dos danos irreversíveis a estas organelas. Estas alterações mitocondriais também foram observadas nas células dos tecidos placentários.

Outros grupos têm analisado as alterações ultraestruturais decorrentes da infecção por ZIKV em diferentes linhagens como as neuronais humanas, hepáticas, células de rim de macaco *Rhesus*, ou células de mosquito, nas quais identificaram alterações semelhantes no retículo endoplasmático, formação de vesículas e detecção de partículas virais de tamanho similar ao que encontramos, também dispostas em aglomerados (312–314). Trabalhos

realizados em diferentes tipos de células infectadas com vírus da dengue também mostram esses tipos de alterações nas organelas, principalmente no retículo endoplasmático e nas mitocôndrias (315–318).

Como observamos a lesão mitocondrial na ultraestrutura das células infectadas por ZIKV e levando em consideração que outros flavivírus, como dengue, causam estresse oxidativo nas células infectadas, investigamos se o ZIKV poderia levar ao mesmo tipo de dano nas células trofoblásticas (170). O metabolismo celular produz ROS normalmente, como subproduto do metabolismo aeróbico normal, por uma variedade de enzimas em mitocôndrias, retículos endoplasmáticos e peroxisomos, e simultaneamente os ânions superóxido são removido para manter o equilíbrio (170). O estresse oxidativo resulta de um desequilíbrio entre as ROS, e as espécies químicas pró-oxidantes e antioxidantes, que levam a danos oxidativos celulares, sendo as mitocôndrias o principal local de produção de ROS (170,319).

As enzimas endógenas fazem parte dos sistemas antioxidantes nas células e, em nosso estudo, avaliamos a atividade das enzimas SOD e CAT. A SOD é responsável pela conversão dos ânions superóxido (produzidos por um vazamento de elétrons da cadeia de transporte de elétrons) em peróxido de hidrogênio e sua atividade foi significativamente aumentada nas células infectadas por ZIKV (170,320). Esse era um resultado esperado, como conseqüência do dano mitocondrial e superprodução de ânions superóxido. A CAT é a enzima que atua em sequência, convertendo o peróxido de hidrogênio em oxigênio e água. Frequentemente, a atividade de CAT é inversa a de SOD, e não foi diferente nas nossas análises. Houve uma diminuição de sua atividade, provavelmente devido à sua disponibilidade dentro da célula, que é menor que a de SOD e devido ao rápido consumo e exaustão do armazenamento dessas enzimas (321–323).

Outra evidência do dano causado às células da placenta pela infecção foi o acúmulo de MDA, que é um produto da peroxidação de ácidos graxos (173). Apesar do dano causado pelo MDA ainda não ter sido totalmente elucidado, sabe-se que essa molécula é capaz de interagir com as bases nitrogenadas dos ácidos nucleicos para formar vários adutos diferentes e causar mutações, levando à danos graves no DNA e RNA celular (173,324).

Por fim, observamos um aumento significativo de óxido nítrico nas células infectadas. O óxido nítrico é um gás solúvel amplamente conhecido por seu papel no controle da circulação e na modulação da vasodilatação, bem como nas sinapses e na inflamação (172,325,326). Sua capacidade de ser benéfico ou potencialmente tóxico por causa da concentração ou purificação no tecido faz com que seja uma molécula complexa e antagônica. A alta concentração dessa espécie reativa de nitrogênio é potencialmente tóxica, pois sua

reação com os ânions superóxido gera o peroxinitrito, que é altamente reativo e pode causar sérios danos no interior da célula, bem como no ambiente externo, uma vez que é lipossolúvel (172).

Infecções com outros flavivírus, como o da dengue, desencadeiam o estresse oxidativo, que afeta tanto o metabolismo celular como o ciclo de vida do próprio vírus (170). Em outros tipos celulares, já foi observado que o estresse oxidativo causado pelo ZIKV leva a alterações na tradução do RNA produzido pela célula e consequentemente à montagem de grandes agregados, compostos pelos complexos de pré-iniciação de tradução interrompidos, chamados grânulos de estresse, além de desencadear o processo de morte celular (174,175). Estudos que investigam o estresse oxidativo devido ao ZIKV ainda são escassos e mais estudos devem ser realizados para uma maior compreensão das conseqüências desse vírus, especialmente nas células da placenta (170).

Através dos nossos achados, podemos dizer que as células placentárias são permissivas à infecção por ZIKV, o que sugere que os trofoblastos contribuem para a transmissão vertical. A análise ultraestrutural das células HTR8/SVneo infectadas com o ZIKV apresentou alterações morfológicas e paralelamente, foi observado o perfil das alterações bioquímicas das células infectadas, indicando estresse oxidativo. Estes resultados irão ajudar a compreender melhor a patogênese da doença, elucidando as consequências da infecção nas células placentárias humanas.

CONCLUSÕES

Com os estudos desta tese podemos concluir:

a) que observamos que diferentes células placentárias, assim como as células imunes residentes ou circulantes são suscetíveis e permissivas a infecção por ZIKV tanto *ex vivo* quanto *in vitro*, o que pode ratificar a hipótese de transmissão vertical célula a célula;
b) a infecção por ZIKV parece ser persistente no tecido placentário e algumas vezes a viremia pode ser persistente, já que houve detecção da replicação no tecido e detecção do RNA viral no sangue de algumas pacientes após meses da fase aguda da doença;
c) houve diferentes lesões histológicas nas placentas e órgãos do natimorto causadas pela infecção por ZIKV, que não são patognomônicas;
d) observamos muitas modificações nos aspectos ultraestruturais das células infectadas, tanto no tecido placentário quanto nas culturas *in vitro* de mastócitos e células trofoblásticas;
e) o aumento na quantidade de células imunes, citocinas e mediadores pró-inflamatórios nas placentas caracterizam um perfil inflamatório local causado pela infecção, com aumento da permeabilidade vascular e facilitação da dispersão viral;
f) a inflamação não parece ter um perfil determinante para o desenvolvimento de microcefalia fetal, em contrapartida, a expressão do fator neutrófico BDNF pode ser fundamental para o desenvolvimento do sistema nervoso central fetal;
g) a dispersão de ZIKV para os tecidos fetais do natimorto com 15 semanas sugere que a infecção pode ter sido a causa do aborto espontâneo e letalidade para o feto;
h) detectamos pela primeira vez a replicação de ZIKV em mastócitos placentários e *in vitro*, observamos que o vírus induz degranulação, produção de citocinas e fatores que tem um papel na inflamação e permeabilidade vascular;

i) a infecção pelo ZIKV nas células trofoblásticas *in vitro* levou ao estresse oxidativo dessas células, com alterações nas enzimas antioxidantes e espécies reativas.

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APÊNDICE A – Artigos publicados durante o período do Doutorado

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Data in Brief

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Data Article

Dataset of proteins mapped on HepG2 cells and those differentially abundant after expression of the dengue non-structural 1 protein

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The data supplied in this article are related to the research article entitled "The effect of the dengue non-structural 1 protein expression over the HepG2 cell proteins in a proteomic approach" (K. Rabelo, M.R. Trugillo, S.M. Costa, B.A. Pereira, O.C. Moreira, A.T. Ferreira et al., 2016) [1]. The present article provides the inventory of peptides and proteins mapped in a hepatocyte cell line (HepG2) by mass spectrometry in the presence of the non-structural pro- tein 1 (NS1) of Dengue 2 virus (DENV2). Cells were transfected with pcENS1 plasmid, which encodes the DENV2 NS1 protein, or the controls pcDNA3 (negative control) or pMAXGFP, encoding the

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The effect of the dengue non-structural 1 protein expression over the HepG2 cell proteins in a proteomic approach

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abstract

Dengue is an important mosquito borne viral disease in the world. Dengue virus (DENV) encodes a polyprotein, which is cleaved in ten proteins, including the non-structural protein 1 (NS1). In this work, we analyzed the effect of NS1 expression in one hepatic cell line, HepG2, through a shotgun proteomic approach. Cells were transfected with pcENS1 plasmid, which encodes the DENV2 NS1 protein, or the controls pcDNA3 (negative control) and pMAXGFP (GFP, a protein unrelated to dengue). Expression of NS1 was detected by immunofluorescence, western blot and flow cytometry. We identified 14,138 peptides that mapped to 4,756 proteins in all analyzed conditions. We found 41 and 81 differentially abundant proteins when compared to cells transfected with plasmids pcDNA3 and pMAXGFP, respectively. Besides, 107 proteins were detected only in the presence of NS1. We identified clusters of proteins involved mainly in mRNA process and viral RNA replication. Down regulation expression of one protein (MARCKS), identified by the proteomic analysis, was also confirmed by real time PCR in HepG2 cells infected with DENV2. Identification of proteins modulated by the presence of NS1 may improve our understanding of its role in virus infection and pathogenesis, contributing to development of new therapies and vaccines.

Biological significance: Dengue is an important viral disease, with epidemics in tropical and subtropical regions of the world. The disease is complex, with different manifestations, in which the liver is normally affected. The NS1 is found in infected cells associated with plasma membrane and secreted into the circulation as a soluble multimer. This protein is essential for virus viability, although its function is not elucidated. Some reports indicate that the NS1 can be used as a protective antigen for the development of a dengue vaccine, while others suggest its involvement in viral pathogenesis. In this work, we report an in-depth comprehensive proteomic profiling resulting from the presence of NS1 in HepG2 cells. These results can contribute to a better understanding of the NS1 role during infection.

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1. Introduction

Dengue ranks as one of the most important mosquito borne viral disease in the world. It is estimated that 390 million dengue virus (DENV) infections occur yearly worldwide, with 500,000 severe cases and 25,000 deaths, mostly among children [1]. Infection with DENV can result in an extensive spectrum of effects, including a self-limiting acute febrile illness, the dengue fever (DF), which may evolve to severe disease forms, as the dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), with homeostatic and vascular permeability defects [2,3]. The liver is one of the major organ targets in DENV infection and hepatic lesions as necrosis, steatosis, hemorrhage and edema are crucial damages seen in this virus infection [4–7]. Analysis of tissue samples obtained from human dengue fatal cases or infected mice indicates that the liver is a potential target of DENV replication, with detection of virus antigens as well as the negative- RNA stranded in hepatocytes [4,6,8–10].

Dengue is an enveloped virus and belongs to the *Flaviviridae* family, consisting of four serotypes (DENV1-4). The DENV genome is constituted of a single positive RNA strand of nearly 11 kb, which encodes a polyprotein translated during infection. The polyprotein is processed by viral and cellular proteases to yield three structural proteins, capsid

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Research Article

Evidence for Tissue Toxicity in BALB/c Exposed to a Long-Term Treatment with Oxiranes Compared to Meglumine Antimoniate

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Leishmaniasis remains a serious public health problem in developing countries without effective control, whether by vaccination or chemotherapy. Part of the failure of leishmaniasis control is due to the lack of new less toxic and more effective drugs able to eliminate both the lesions and the parasite. Oxiranes derived from naphthoquinones now being assayed are promising drugs for the treatment of this group of diseases. The predicted pharmacokinetic properties and toxicological profiles of epoxy- -lapachone and epoxymethoxy-lawsone have now been compared to those of meglumine antimoniate, and histological changes induced by these drugs in noninfected BALB/c mice tissues are described. Effects of these compounds on liver, kidney, lung, heart, and cerebral tissues of healthy mice were examined. The data presented show that both these oxiranes and meglumine antimoniate induce changes in all BALB/c mice tissues, with the lung, heart, and brain being the most affected. Epoxymethoxy-lawsone was the most toxic to lung tissue, while most severe damage was caused in the heart by epoxy- -lapachone. Meglumine antimoniate caused mild-to-moderate changes in heart and lung tissues.

1. Introduction

Leishmaniasis represents a group of parasitic diseases caused by more than 20 *Leismania* spp. parasites, which are trans-mitted to humans by the bite of infected female phlebotomine sandflies. The epidemiology of leishmaniasis depends on the

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Artigo de Revisão

DESDOBRAMENTOS DOS SURTOS DE INFECÇÃO PELO VÍRUS ZIKA

CONSEQUENCES OF ZIKA VIRUS INFECTION OUTBREAKS

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Resumo: O vírus Zika (ZIKV) é um vírus emergente transmitido por artrópodes que apenas recentemente foi exposto como uma ameaça substancial à saúde pública. O surto de ZIKV no Brasil, especialmente, e sua associação com anormalidades fetais têm levado a preocupação mundial. Esta revisão propõe trazer ao leitor as últimas consequências no mundo e os esforços da comunidade científica para elucidar melhor a transmissão e a patogenia desta doença. Aqui, resumimos esses estudos, incluindo a epidemiologia ZIKV, aspectos virais, transmissão, características e estudos sobre o possível desenvolvimento de novas terapias e vacinas. Palavras Chaves: Infecção, Saúde Pública, Epidemiologia.

Abstract: The Zika virus (ZIKV) is an emerging arthropod-borne virus has only recently been exposed as a substantial public health threat. The outbreak of the ZIKV in Brazil, specially, and its association with fetal abnormalities have raised worldwide concern. This review seeks to bring the reader the latestconsequences in the world and efforts of the scientific community to elucidate better the transmission and pathogeny of this disease. Here, we summarized these studies, including the ZIKV epidemiology, viral aspects, transmission, features and studies about possible development of new therapies and vaccines. Keywords: Infection, Public Health, Epidemiology.

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Research Article

Capybara Oil Improves Hepatic Mitochondrial Dysfunction, Steatosis, and Inflammation in a Murine Model of Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is recognized as the most common cause of liver dysfunction worldwide and is commonly associated with obesity. Evidences suggest that NAFLD might be a mitochondrial disease, which contributes to the hepatic steatosis, oxidative stress, cytokine release, and cell death. Capybara oil (CO) is a rich source of polyunsaturated fatty acids (PUFA), which is known to improve in ammation and oxidative stress. In order to determine the e ects of CO on NAFLD, C Bl/ mice were divided into groups and fed a high-fat diet (HFD) (NAFLD group and NAFLD + CO group) or a control diet (CG group) during 1 weeks. Te CO (1. g/kg/daily) was administered by gavage during the last 4 weeks of the diet protocol. We evaluated plasma liver enzymes, hepatic steatosis, and cytokine expression in liver as well as hepatocyte ultrastructural morphology and mitochondrial function. CO treatment suppressed hepatic steatosis, attenuated in ammatory response, and decreased plasma alanine aminotransferase (ALT) in mice with NAFLD. CO was also capable of restoring mitochondrial ultrastructure and function as well as balance superoxide dismutase and catalase levels. Our ndings indicate that CO treatment has positive e ects on NAFLD improving mitochondrial dysfunction, steatosis, acute in ammation, and oxidative stress.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the accumulation of fat in the liver in patients who do not consume excessive alcohol [1]. Te term NAFLD encompasses a wide spectrum of conditions, from simple accumulation of fat (“fatty liver” or steatosis) to steatohepatitis, brosis, and cirrhosis with its clinical consequences [2]. NAFLD a ects about 0% of the general population in western society and is recognized as the most common cause of liver dysfunction worldwide [, 4].

NAFLD is asymptomatic in most a ected patients and characterized as the presence of more than % of lipid accumulation in the hepatocytes, excluding other liver disease etiologies (virus, autoimmune, alcohol, drugs, and genetics). NAFLD can progress to an advanced form, nonalcoholic steatohepatitis (NASH), which is de ned by histological

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ARTICLE

Evaluation of different transfection methodologies to achieve efficient expression of the NS1 dengue protein in HepG2 cells

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HepG2, a human hepatocarcinoma cell line, has been used as a model to study infection by several pathogens including dengue virus. However, this cell line is notoriously difficult to be transfected with plasmid DNAs by traditional methods, which is a limitation for some studies involving heterologous gene expression. In the present work, we analyzed different protocols for transfection of HepG2 with the plasmid pcENS1, which encodes the dengue NS1 protein, in order to evaluate the best methodology for achieving high cell viability and transfection efficiency. We analyzed two transfection approaches using lipid-based methods (Lipofectamine and FuGENE 6) or electroporation by nucleofection. Expression of the recom-binant NS1 protein was evaluated by immunofluorescence and flow cytometry. Transfection with either of the two lipid-based methods led to very low number of HepG2 cells expressing NS1 (3.9% and 6.8% with Lipofectamine and FuGene, respectively) and high cell death rates. On the other hand, the efficien-cy of cell transfection was remarkable higher with nucleofection when compared to these other methods, achieving 63% of cells expressing NS1 protein and more than 60% of viability in the optimized condition.

Key words: HepG2, Transfection, Nucleofection, Lipid-based methods, NS1, dengue

INTRODUCTION

High efficiency of DNA delivery and heterologous protein expression are critical factors for molecular and cell biology studies, as for example functional analyses of proteins, their localization and trafficking. In order to introduce DNA into mammalian cell, several methods of *in vitro* transfection are available, such as cationic liposomal reagents (Balazs and Godbey 2011), solid nanomaterials (Unciti-Broceta et al. 2012; Dréan et al. 2017), electroporation (Brunner et al. 2002) and nucleofection (Kraus et al. 2010).

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These techniques aim to overcome the barriers that exist within cells for introduction of a heterologous DNA, such as plasma and nuclear membranes, as well as to allow DNA escape from endosomes. Moreover, the DNA has a polyanionic characteristic and high molecular weight, which hampers passing through cell membranes (Luo and Saltzman 2000). In addition, transfection methods may be cytotoxic with variable rates.

Cationic liposomes are widely used to facilitate DNA transfection because they complex to DNA, neutralizing its charge (Susa, et al. 2008). Alternatively, electroporation destabilizes cell membranes with high intensity electrical pulses, and is considered a simple and efficient technique for introduction of exogenous genes

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BALB/c mice infected with DENV-2 strain 66985 by the intravenous route display injury in the central nervous system

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Dengue is a mild flu-like arboviral illness caused by dengue virus (DENV) that occurs in tropical and subtropical countries. An increasing number of reports have been indicating that dengue is also associated to neurological manifestations, however, little is known regarding the neuropathogenesis of the disease. Here, using BALB/c mice intravenously infected with DENV-2 strain 66985, we demonstrated that the virus is capable of invading and damaging the host's central nervous system (CNS). Brain and cerebellum of infected animals revealed histological alterations such as the presence of inflammatory infiltrates, thickening of pia matter and disorganization of white matter. Additionally, it was also seen that infection lead to altered morphology of neuroglial cells and apoptotic cell death. Such observations highlighted possible alterations that DENV may promote in the host's CNS during a natural infection, hence, helping us to better understand the neuropathological component of the disease.

Dengue is a mosquito-borne disease that represents a major health problem especially in tropical and subtropical regions worldwide. e disease is caused by dengue virus (DENV), which comprises four antigenically di erent serotypes (DENV-1 to DENV-4) belonging to the *Flaviviridae* family. Dengue burden has been expanding since 1960s as it grew side by side with the world's population. Nowadays, around 390 million people are infected every year, of which about 25% are of clinical relevance[1]. Symptoms of dengue are usually similar to the regular u, however, a small fraction of cases may evolve to a severe hemorrhagic form that is eventually responsible for about 20,000 deaths in an annual basis[2,3].

An intriguing fact that has drawn attention in dengue is the involvement of the host's central nervous system (CNS) in the course of infection. CNS-related symptoms of dengue were rst reported as an acute encephalopathy in 1976[4] and, classically, these manifestations have been treated as rare phenomena in humans[5,6]. Back in 1998,

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Case Report

Zika virus found in brain tissue of a multiple sclerosis patient undergoing an acute disseminated encephalomyelitis-like episode

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Abstract

Background: A range of different neurological manifestations has been reported in fetuses and adults after Zika virus (ZIKV) infection.

Objective: We describe a detection of the ZIKV in the brain tissue from a multiple sclerosis (MS) patient with acute disseminated encephalomyelitis (ADEM)-like event in Rio de Janeiro, Brazil.

Methods: Biological samples collected during the hospitalization were tested by serology and molecular diagnostic for various infectious agents. Histopathological analysis was performed using the anti-flavivirus group 4G2 monoclonal antibody, anti-ZIKV non-structural 1 (NS1) monoclonal antibody, and anti-CD4, CD8, and CD11b antibodies.

Results: Anti-ZIKV IgM and IgG antibodies were positive in the serum and urine. A brain biopsy showed ZIKV protein in brain cells and T CD8 infiltration in brain tissue.

Conclusion: Our data describe the coexistence of a recent central nervous system (CNS) ZIKV infec-tion accompanied by a severe ADEM-like syndrome outcome in a patient with clinical history of MS. A de novo immune response concomitant with ZIKV infection might be involved in the mechanism of the ADEM-like syndrome and response to immunotherapy. The present report reinforces the importance of providing the differential diagnosis of acute episodes of MS exacerbation in an environment prone to ZIKV expression.

Keywords: Multiple sclerosis, Zika virus, acute disseminated encephalomyelitis, differential diagnosis, immunotherapy

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Introduction

Multiple sclerosis (MS) is an inflammatory and autoimmune disease of the central nervous system (CNS).[1] Acute disseminated encephalomyelitis (ADEM) is an acute inflammatory demyelinating disease that, as MS, predominantly affects the white matter and occurs with a temporal relationship to post-infection or post-vaccination.[2]

Zika virus (ZIKV) targets human brain cells, provokes an immune activation, and in contrast with asymptomatic patients, reports of neurological disorders are usually severe.[3,4] Here, we provide evidence of brain exposure to ZIKV in the case of a Brazilian MS patient confirmed infected with ADEM-like syndrome and the diagnostic challenge during the MS course and treatment.

Case report

A 35-year-old female was diagnosed with MS in 2012. MS symptoms were a cervical myelitis, and a magnetic resonance imaging (MRI) examination showed a T2 weighted-image (WI) hyperintense

SCIENTIFIC REPORTS

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Dengue virus nonstructural 3 protein interacts directly with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and reduces its glycolytic activity

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Dengue is an important mosquito-borne disease and a global public health problem. The disease is caused by dengue virus (DENV), which is a member of the Flaviviridae family and contains a positive single-stranded RNA genome that encodes a single precursor polyprotein that is further cleaved into structural and non-structural proteins. Among these proteins, the non-structural 3 (NS_3) protein is very important because it forms a non-covalent complex with the NS_2B cofactor, thereby forming the functional viral protease. NS_3 also contains a C-terminal ATPase/helicase domain that is essential for RNA replication. Here, we identified 47 NS_3-interacting partners using the yeast two-hybrid system. Among those partners, we highlight several proteins involved in host energy metabolism, such as apolipoprotein H, aldolase B, cytochrome C oxidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH directly binds full-length NS_3 and its isolated helicase and protease domains. Moreover, we observed an intense colocalization between the GAPDH and NS_3 proteins in $DENV_2$-infected Huh7.5.1 cells, in NS_3-transfected BHK-21 cells and in hepatic tissue from a fatal dengue case. Taken together, these results suggest that the human GAPDH-DENV NS_3 interaction is involved in hepatic metabolic alterations, which may contribute to the appearance of steatosis in dengue-infected patients. The interaction between GAPDH and full-length NS_3 or its helicase domain *in vitro* as well as in NS_3-transfected cells resulted in decreased GAPDH glycolytic activity. Reduced GAPDH glycolytic activity may lead to the accumulation of metabolic intermediates, shifting metabolism to alternative, non-glycolytic pathways. This report is the first to identify the interaction of the $DENV_2$ NS_3 protein with the GAPDH protein and to demonstrate that this interaction may play an important role in the molecular mechanism that triggers hepatic alterations.

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VIRUSES

MDPI

Article

A Stillborn Multiple Organs' Investigation from a Maternal DENV-4 Infection: Histopathological and Inflammatory Mediators Characterization

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Abstract: Dengue virus (DENV) is an emerging virus involved in outbreaks in Brazil. The association between the virus and vertical transmission, with disorders in the placenta, has raised a worldwide concern. On the 29th gestational week, a pregnant woman presented severe complications due to a DENV infection leading to maternal and fetus death. Postmortem analysis of fetal organs demonstrated the presence of DENV using reverse transcriptase polymerase chain reaction (RT-PCR) in the fetal brain and DENV non-structural protein 3 (NS3) staining in placenta and several peripheral fetal tissues, such as the brain, liver, lungs, and spleen. Histological analysis of the placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema, and necrosis in placenta and tissue disorganization in the fetus, such as spongiform parenchyma, microglial inflammation, steatosis, hyalinose arteriolar, inflammatory cells in the alveolar septa, and disorganization of the lymphoid follicle. Increased cellularity (macrophage, Hofbauer cells and TCD8+ lymphocytes) and up-regulation of inflammatory mediators such as IFN- , TNF- , RANTES/CCL5, MCP1/CCL2, and VEGF/R2 were detected in the liver, lung, spleen, brain, and placenta, supporting placental and fetus peripheral tissues inflammation. Maternal infection leading to the production of those vascular mediators may alter the vascular permeability, facilitating the virus entry and tissue and barrier dysfunction.

Keywords: dengue 4; pregnancy; fetal death; cytokines; inflammatory mediators

Therefore, we aimed to analyze uvaol effects in trophoblast cells incubated with inactivated GBS.
Methods: HTR-8/SVneo cells were treated with uvaol and/or inactivated serotype Ia GBS. Cell viability was measured by MTT assay and cell death by flow cytometry analyses of Annexin V / Propidium Iodide staining. Nitrite production was evaluated by Griess reaction and oxygen reactive species by DCFH assay. Nuclear translocation of NFkB p65 was detected by immunofluorescence, and Th1/Th2 cytokines production was measured by RT-PCR and their secretion by flow cytometry using bead-based multiplex assay.

Results: Uvaol treatment protected cells against viability loss and cell death caused by GBS at 10^8 CFU. Oxygen and nitrogen reactive species were unchanged by uvaol. Nuclear translocation of NFkB p65 began 15 minutes after GBS incubation and uvaol inhibited this process. GBS decreased IL-4 secretion and increased IL-1b, IFN-g and IL-2, while uvaol prevented the IL-1b, IFN-g and IL-2 increase induced by GBS.

Conclusion: Uvaol is an interesting anti-inflammatory natural product that could prevent trophoblast cell death and inflammation caused by inactivated-GBS, and might be useful against GBS deleterious effects on pregnancy.

P1.77.
ZIKA VIRUS AND PLACENTA: HISTOPATHOLOGICAL INVESTIGATION, INFLAMMATORY PROFILE AND ULTRASTRUCTURAL ANALYSIS IN GESTATIONS RESULTING IN CASES OF MICROCEPHALY OR NOT

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Objectives: In this work, we aimed to investigate the histopathological and ultrastructural changes, and the immunological profile in the placenta of 10 Zika virus (ZIKV)-infected patients during pregnancy, 5 pregnancies resulting in cases of microcephaly and 5 non-microcephaly compared to 5 non-infected control placentae.

Methods: Clinical and laboratory examinations of the pregnant women were accomplished. Histopathology by H.E. and Picro Sirius Red stainning, ZIKV immunoassays, and ultrastructural evaluation of the placenta were performed.

Results: The evaluation of the abnormally large term placenta revealed severe damage to the maternal decidua and chorionic villi. Maternal por-tions presented diffuse edema, fibrinoid necrosis, fibrosis, degeneration, calcification, and focal areas of inflammatory infiltrates. An investigation of the chorionic villi presented an area of extensive calcification, vascular endothelial thickening, and perivascular inflammatory infiltrates. These features were not observed in the controls placentae. Additionally, there was an expressive decrease of collagen (up to 60%) in infected placentas. ZIKV-E and NS1 protein were detected only in samples from the ZIKV infected patients by immunohistochemistry. Increased cellularity (Hofba-uer cells and $TCD8^+$ lymphocytes), expression of MMP-2 and MMP-9, as well as local proinflammatory cytokines such as IFN-g and TNF-a, and other markers such as RANTES / CCL5 and VEGFR2, confirm inflammation and placental dysfunction. The infection of Hofbauer cells during gestation not only reflects the critical failure of the maternal-fetal protection arrangement, but also highlights a potential pathway for the vertical transmission of ZIKV. Ultrastructural aspects of this sample showed endothelium thickening, damaged syncytiotrophoblasts nuclei, rarefied cytoplasm, abnormal organelles and intracellular clusters of virus-like particles approximately 25 nm in diameter.

Conclusion: The placental changes caused by ZIKV are not pathogno-monic, however, we provide evidence that this infection leads to severe placental injury to the maintenance of pregnancy, and these results sup-port the understanding of the Zika disease's immunopathogenesis.

P1.78.
DIFFERENTIAL SECRETION OF INFLAMMATORY CYTOKINES BY HUMAN TROPHOBLAST IN THE PRESENCE OF E. COLI AND TWO LACTOBACILLUS STRAINS.

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Objectives: To compare the resulting cytokine pattern after in vitro interaction of Sw.71, an immortalized human first trimester trophoblast cells and two lactobacillus strains and one common urogenital pathogenic bacterium.

Methods: Sw.71 were incubated with 5×10^5 E. coli (ATCC 700926),or L. jensenii (ATCC 25258) or L. crispatus isolated from human vaginal swabs, during six hours. Conditioned media were analyzed with a human cyto-kine/chemokine magnetic bead assay kit (Milliplex MAG, Millipore, MA, USA).Two-way analysis of variance was used to compare cytokines/che-mokines concentrations in the culture media.

Results: Sw.71 co-incubated with E. coli, L. crispatus or L. jensenii resulted in differential secretion of 11 of the 26 assayed cytokines/chemokines. Sw.71 co-incubated with any of the three bacteria responded with sig-nificant increased secretion of IL-8, and GMCSF. On the other hand, MCP-1 was only significantly stimulated in the presence of both lactobacilli strains. A general tendency was that in the presence of both lactobacilli, secretion of pro-inflammatory cytokines by Sw.71 was decreased. Sw.71 cells responded to co-incubation with both lactobacilli strains secreting increased levels of IL-10 and IL-1ra, nor with E. coli.

Conclusion: We provide evidence that human trophoblast can react differentially to pathogenic bacteria (E. coli) than to normal components of the cervicovaginal microbiota (lactobacilli). Our results support the exis-tence of separated mechanisms of bacteria recognition by the trophoblast or presence of bacterial products leading to alternative inflammatory/anti-inflammatory responses that are coincident with intrauterine tolerance to lactobacilli. Sw.71 cells reacted to lactobacilli with a combination of increased secretion of chemokines, increased secretion of anti-inflamma-tory cytokines and low secretion of pro-inflammatory signals. More intriguing is the role of the trophoblast in the cross-talk with normal components of the reproductive tract microbiota such as, L. crispatus and L. jensenii.

P1.79.
CHRONIC INTERVILLOSITIS LINKED TO PLATELET ALLOIMMUNIZATION e A NEW ENTRY PORT TO UNDERSTANDING A RARE BUT IMPORTANT PLACENTAL LESION

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Student's t-test were used to compare treated explants with controls.
Significance was set at p-value <0.05.
Results: L-1MT blocked specific IDO activity, decreasing the Kyn levels (0.5643±0.04 vs 1.158±0.135 relative to control) and increasing parasitic load in infected explants (2.086±0.371 vs 1.00±0.001 relative to control). IFN-ɣ increased the production of L-Kyn (1.903±0.095 vs 1.158±0.135 relative to control). L-Trp increased specific IDO activity (1.678±0.237 vs 0.095 relative to control) and Kyn levels in supernatants (5.933±0.7058 vs 1.691±0.482 relative to control). Treatment with L-Trp decreased parasitic load in infected explants (0.228±0.051 vs 1.00±0,001 relative to control). 3-HK and 3-HAA decreased parasitic load when compared to non-treated explants (0.422±0.0494 vs 1.00±0.001; 0.596±0.072 vs 1.00±0,001 relative to control).

Conclusion: Catabolism of Trp participates in T. cruzi infection process through some metabolites (3-HK and 3.HAA) of the chorionic villi KP, regulating the load of the placental infection, which would be the first step to congenital transmission of Chagas disease.
Keywords: T. cruzi, Human placental, L-Tryptophan, Kynurenine, 3-HK, 3-HAA

P2.73.
EFFECTS OF THE E.COLI EXOTOXIN ALPHA-HEMOLYSIN ON HUMAN CHORIOAMNIOTIC MEMBRANES.

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Objectives: Premature rupture of chorioamniotic membranes is associated to urinary tract infections in pregnant women. Therefore, the main aim of this study is to evaluate the effect of alpha-hemolysin (HlyA), an exotoxin secreted by uropathogenic E.Coli, in remodeling of chorioamniotic extra-cellular matrix in vitro.

Methods: HlyA was purified from a WAM1824 E.coli culture by isoelectric precipitation. Chorioamniotic membranes were obtained from deliveries by elective cesarean section (>37 weeks). All included women had uneventful preg-nancies, without evidence of active labor and with no signs of infection.
Membrane discs (8mm) were cultured in presence of 0-50nM HlyA for 3 and 24h. We evaluated membrane integrity by LDH and MTT assays and Masson's trichrome stain to stablish work conditions (n¼3).
Then, explants (18mm) were cut to be placed on a Transwell system to form two independent chambers and insured with linen thread. To simulate an ascending infection, explants were incubated in control con-ditions, with 5nm/50nm HlyA, and 50nM pro-HlyA (inactive form) in the chorion-side during 24h, (n¼3).

We evaluated membrane integrity by hematoxylin-eosin staining on paraffin-embedded tissue sections and LDH assay on culture medium. Collagen levels were tested by Masson's trichrome stain, metalloprotease (MMP) activity by zimography, and cyclooxygenase-2 (COX2) expression by RT-qPCR.

Results: Untreated discs remain intact in culture until 48h. Instead, explant of fetal membrane incubated with 5 and 50nM HlyA during 24h showed a detrimental effect in mitochondrial activity and collagen level without LDH release.

HlyA induced a structural alteration and a decrease in collagen fibers content in mounted explants. Moreover, an increment in COX-2 expression and metalloproteinase activity were observed in a HlyA concentration dependence. Instead, Pro-HlyA-treated explants remained similar to control.

Conclusion: E.Coli HlyA toxin by itself is capable to trigger events related to remodeling of chorioamniotic extracellular matrix in vitro. This observa-tion suggets that the blockeage of HlyA may contribute to diminish dam-age on fetal membrane.

P2.74.
PLACENTAL INJURY OF A PREGNANT WOMAN INFECTED WITH CHIKUNGUNYA IN THE THIRD TRIMESTER OF GESTATION.

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Objectives: The objective of this work is to describe a case of a 40-year-old, pregnant woman (36 weeks of gestation) from Campos dos Goytacazes, State of Rio de Janeiro, Brazil, gave birth in May 2018, at the Hospital Plantadores de Cana. She reported the onset of symptoms such as head-ache, intense arthralgia and exanthema, three days before the day of delivery.

Methods: After her consent, serum and placenta were collected and sent to Fiocruz for further investigations. The serology, qRT-PCR, histopatho-logical analysis, immunhistochemistry and electron microscopy were performed to investigate Chikungunya infection.
Results: IgM antibodies against CHIKV were detected and RT-qPCR of the placenta was positive for CHIKV. The histopathological analysis exhibited mononuclear cells infiltrate, delayed villous maturation, deciduitis, decidual necrosis and extramedullary hematopoiesis. Moreover, immu-nohistochemistry assay revealed CHIKV positivity in extramedullary cells in fetal capillaries, in villi and syncytiotrophoblast cells

Conclusion: These findings suggest that CHIKV infects the placenta at the third trimester of gestation, which could lead to alterations in its structure, and consequently may impair its functionality. Although the newborn did not develop disease, it seems that there was a fetal response. Further studies is required to understand the immunopathology of CHIKV, and which consequences of its infection during pregnancy.

P2.75.
THE IMMUNOSUPPRESSANT DRUG AZATHIOPRINE INDUCES EXPRESSION OF IL-18 IN PLACENTAL CELLS

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Objectives: In vitro analysis of protein expression of NLRP3, IL-1ß, and IL-18 in full-term human chorionic villous explants treated with the immu-nosuppressant drug azathioprine
Methods: Chorionic villi were isolated from healthy-term pregnancies placentas obtained following Caesarean section. The villi were cultured in DMEM/F12 medium with 10% heat-inactivated fetal bovine serum, in standard culture conditions. In time-course and concentration-response experiments, placental villi were incubated for 6, 12, or 24 h with 0, 10 and 100 ng/mL of the immunosuppressive drug Azathioprine (AZA). The pro-tein expression of NLRP3, IL-1ß, and IL-18 was assessed by Western blot-ting. The viability was determined by lactic dehydrogenase (LDH) assay.

Results: The viability did not change significantly, except for 100 ng/mL AZA, but the expression of IL-18 increased markedly in a concentration-dependent manner. The treatment also increased placental IL-1ß and NLRP3, but not at the same levels of IL-18

Conclusion: Our preliminary data show that AZA may be toxic in high concentration as evidenced by LDH damage to placental cells. Further-more, our results also suggest AZA may contribute to NLRP3 inflamma-some activation and overexpression of IL-18 in placental villi.
As an IFN-gamma inducing factor, IL-18 may be responsible for altering the maternal cytokine balance in transplanted recipient pregnant women, which may be associated with several gestational consequences and out-comes.

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Histology and Histopathology

From Cell Biology to Tissue Engineering

Aliskiren improves renal morphophysiology and inflammation in Wistar rats with 2K1C renovascular hypertension

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Summary. Hypertension is characterized by persistent elevated blood pressure levels, one of the leading causes of death in the world. Renovascular hypertension represents the most common cause of secondary hypertension, and its progress is associated with overactivation of the renin angiotensin aldosterone system (RAAS), causing systemic and local changes. Aliskiren is a renin-inhibiting drug that optimizes RAAS suppression. In this sense, the objective of the present study was to analyze the morphophysiology of the left kidney in Wistar rats with renovascular hypertension after treatment with Aliskiren. Parameters such as systolic blood pressure, urinary creatinine and protein excretion, renal cortex structure and ultrastructure, fibrosis and tissue inflammation were analyzed. Our results showed that the hypertensive animals treated with Aliskiren presented a reestablishment of blood pressure, expression of renin, and renal function, as well as a remodeling of morphological alterations through the reduction of fibrosis. The treatment regulated the laminin expression and decreased pro -inflammatory cytokines, restoring the integrity of the glomerular filtration barrier. Therefore, our findings suggest that Aliskiren has a renoprotective effect acting on the improvement of the morphology, physiology and pathology of the renal cortex of animals with renovascular hypertension.

Keywords: Renovascular hypertension, Aliskiren, Wistar, Inflammation, Renal histopathology

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Introduction

Systemic arterial hypertension is a multifactorial clinical condition characterized by persistent elevated blood pressure levels. The death rate caused by hypertension increased 13.2% within a period of ten years in over 190 countries (Sociedade Brasileira de Cardiologia, 2010). Secondary hypertension is characterized by the existence of an unknown triggering factor, in which renovascular disease is one of the most common causes of secondary hypertension (Pullalarevu et al., 2014) . Renovascular hypertension (RH) is described as a partial reduction of renal perfusion pressure, caused by a stenotic or obstructive lesion of one or both renal arteries, leading to an increase in the activity of the renin angiotensin aldosterone system (RAAS) (Ledingham, 1971).

The most used experimental model for the study of the pathogenesis of RH is the prototype of Goldblatt, with the reduction of the renal blood supply. In this model, called 2 Kidney - 1 Clip (2K1C), HR is induced by unilateral partial occlusion, by implantation of a silver clip in the renal artery, causing a reduction in renal blood by approximately 50% and promoting a necessary

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Dengue infection in mice inoculated by the intracerebral route: neuropathological effects and identification of target cells for virus replication

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Dengue is an important arboviral infection, causing a broad range symptom that varies from life-threatening mild illness to severe clinical manifestations. Recent studies reported the impairment of the central nervous system (CNS) after dengue infection, a characteristic previously considered as atypical and underreported. However, little is known about the neuropathology associated to dengue. Since animal models are important tools for helping to understand the dengue pathogenesis, including neurological damages, the aim of this work was to investigate the effects of intracerebral inoculation of a neuroadapted dengue serotype 2 virus (DENV2) in immunocompetent BALB/c mice, mimicking some aspects of the viral encephalitis. Mice presented neurological morbidity after the 7th day post infection. At the same time, histopathological analysis revealed that DENV2 led to damages in the CNS, such as hemorrhage, reactive gliosis, hyperplastic and hypertrophied microglia, astrocyte proliferation, Purkinje neurons retraction and cellular infiltration around vessels in the pia mater and in neuropil. Viral tropism and replication were detected in resident cells of the brain and cerebellum, such as neurons, astrocyte, microglia and oligodendrocytes. Results suggest that this classical mice model might be useful for analyzing the neurotropic effect of DENV with similarities to what occurs in human.

Dengue is one of the most important diseases caused by an arbovirus, the dengue virus (DENV), which a ects 96 million people annually worldwide, with 396 million estimated infections[1]. e disease has a broad range manifestation, varying from a life-threatening mild u-like illness, known as dengue fever, to severe dengue[2]. e virus belongs to the family *Flaviviridae*, genus *Flavivirus*, and consists of four antigenically distinct serotypes (DENV 1–4)[3].

Although DENV is classically characterized as a non-neurotropic virus, in the last decades several studies led to a di erent understanding of the clinical pro le of the dengue disease. In addition to a variety of non-speci c signs and symptoms, the disease may also present manifestations including neurological involvements[4]. e emergence of neurological signs in human patients infected with DENV was rst reported in 1976[5]. Nowadays, encephalitis and encephalopathy stand out as the most common neurological signs resulting from DENV infection[6].

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Renal Injury in DENV-4 Fatal Cases: Viremia, Immune Response and Cytokine Profile

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Abstract: Dengue virus (DENV) infections may result in asymptomatic cases or evolve into a severe disease, which involves multiple organ failure. Renal involvement in dengue can be potentially related to an increased mortality. Aiming to better understand the role of DENV in renal injury observed in human fatal cases, post-mortem investigations were performed in four DENV-4 renal autopsies during dengue epidemics in Brazil. Tissues were submitted to histopathology, immunohistochemistry, viral quantification, and characterization of cytokines and inflammatory mediators. Probably due the high viral load, several lesions were observed in the renal tissue, such as di use mononuclear infiltration around the glomerulus in the cortical region and in the medullary vessels, hyalinosis arteriolar, lymphocytic infiltrate, increased capsular fibrosis, proximal convoluted tubule (PCT) damage, edema, PCT debris formation, and thickening of the basal vessel membrane. These changes were associated with DENV-4 infection, as confirmed by the presence of DENV-specific NS3 protein, indicative of viral replication. The exacerbated presence of mononuclear cells at several renal tissue sites culminated in the secretion of proinflammatory cytokines and chemokines. Moreover, it can be suggested that the renal tissue injury observed here may have been due to the combination of both high viral load and exacerbated host immune response.

Keywords: dengue 4; fatal case; viremia; histopathology; cytokines; inflammatory mediators

viruses

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Article

Fatal Dengue Cases Reveal Brain Injury and Viral Replication in Brain-Resident Cells Associated with the Local Production of Pro-Inflammatory Mediators

Natália Salomão [1], Kíssila Rabelo [2], Carlos Basílio-de-Oliveira [3], Rodrigo Basílio-de-Oliveira [3], Luiz Geraldo [4], Flávia Lima [4], Flávia dos Santos [5], Gerard Nuovo [6,7], Edson R. A. Oliveira [8] and Marciano Paes [1,*]

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Abstract: Dengue is an arboviral disease caused by dengue virus (DENV), which is transmitted to humans by *Aedes aegypti* mosquitoes. Infection by DENV most commonly results in a mild flu-like illness; however, the disease has been increasingly associated with neurological symptomatology. This association draws attention to further investigations on the impact of DENV infection in the host's central nervous system. Here, we analyzed brain samples of three fatal dengue cases that occurred in 2002 during an outbreak in Rio de Janeiro, Brazil. Brain tissues of these cases were marked by histopathological alterations, such as degenerated neurons, demyelination, hemorrhage, edema, and increased numbers of astrocytes and microglial cells. Samples were also characterized by lymphocytic infiltrates mainly composed of CD8 T cells. DENV replication was evidenced in neurons, microglia and endothelial cells through immunohistochemistry and in situ hybridization techniques. Pro-inflammatory cytokines, such as TNF-α and IFN-γ were detected in microglia, while endothelial cells were marked by the expression of RANTES/CCL5. Cytoplasmic HMGB1 and the production of nitric oxide were also found in neurons and microglial cells. This work highlights the possible participation of several local pro-inflammatory mediators in the establishment of dengue neuropathogenesis.

Keywords: dengue; human fatal cases; neuropathogenesis; inflammation; central nervous system

1. Introduction

APÊNDICE B – Capítulos publicados durante o período do Doutorado

Top 10 Contributions on Immunology: 2nd Edition

Chapter 04

Placental Histopathology and Clinical Presentation of Severe Congenital Zika Syndrome in a Human Immunodeficiency Virus-Exposed Uninfected Infant

Kíssila Rabelo[1], Regina Célia de Souza Campos Fernandes[2,3], Luiz José de Souza[2], Thais Louvain de Souza[2,3], Flávia Barreto dos Santos[4], Priscila Conrado Guerra Nunes[4], Elzinandes Leal de Azeredo[4], Natália Gedeão Salomão[5], Gisela Freitas Trindade6, Carlos A Basílio-de-Olivei-ra[7], Jorge José de Carvalho[1], Enrique Medina-Acosta[3]* and Marciano Viana Paes[5]*

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CAPÍTULO 18

PLACENTAL INFLAMMATION AND FETAL INJURY IN A RARE ZIKA CASE ASSOCIATED WITH GUILLAIN-BARRÉ SYNDROME AND ABORTION

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ABSTRACT: Zika virus (ZIKV) is an emerging virus involved in recent outbreaks in Brazil. The association between the virus and Guillain-Barré syndrome (GBS) or congenital disorders has raised a worldwide concern. In this work, we investigated a rare Zika case, which was associated with GBS and spontaneous retained abortion. Using specific anti-ZIKV staining, the virus was identified in placenta (mainly in Hofbauer cells) and in several fetal tissues, such as brain, lungs, kidneys, skin and liver. Histological analyses of the placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema and necrosis in placenta, as well as tissue disorganization in the fetus. Increased cellularity (Hofbauer cells and $TCD8_+$ lymphocytes), expression of local pro-inflammatory cytokines such as IFN-γ and TNF-α, and other markers, such as RANTES/CCL5 and


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CAPÍTULO 3

CAMUNDONGOS BALB/C INFECTADOS COM A CEPA 66985 DO VÍRUS DA DENGUE PELA VIA INTRAVENOSA EXIBE DANO NO SISTEMA NERVOSO CENTRAL

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ANEXO – Parecer do Comitê de Ética em Pesquisa

FUNDAÇÃO OSWALDO CRUZ - FIOCRUZ/IOC

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise histopatológica em placentas e pele de pacientes infectados com Zika e de tecidos de natimortos originário de casos de microcefalia

Pesquisador: Marciano Viana Paes

Área Temática:

Versão: 2

CAAE: 65924217.4.0000.5248

Instituição Proponente: FUNDACAO OSWALDO CRUZ

Patrocinador Principal: FUN CARLOS CHAGAS F. DE AMPARO A PESQUISA DO ESTADO DO RIO DE JANEIRO - FAPERJ

DADOS DO PARECER

Número do Parecer: 2.055.074

Apresentação do Projeto:

O presente estudo terá parceria dos Hospitais: Gamboa, Hospital Geral em Nova Iguaçu, Hospital Plantadores de Cana em Campos dos Goytacazes, UTI neonatal em Campos dos Goytacazes/Macaé. Em todos esses hospitais serão realizados o acompanhamento de diagnóstico clínico e laboratorial, tratamento e prognóstico de gestantes com sintomatologia de ZIKA atendidas pelo Centro de Diagnóstico de Dengue em parceria com os Hospitais Plantadores de Cana,Maternidade Referência do Município de Campos dos Goytacazes, UTI neonatal (CEPLIN) de Campos e Macaé Gamboa e Hospital Geral de Nova Iguaçu. Todas as coletas de pele, placenta e material do natimorto e análises histopatológicas serão realizadas com a colaboração do Médico patologista Prof. Dr. Carlos Alberto Basílio de Oliveira/da anatomia patológica do Hospital Gafrée Guinle/UNIRIO. As amostras de pacientes e órgãos provenientes de biopsias e necropsias dos casos fatais de Zika serão armazenadas no laboratório Interdisciplinar de Pesquisas Médicas. Fragmentos de tecidos placentas e biópsias de pele serão coletados para observação em microscopia óptica e eletrônica (M.E.).

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FACULDADE DE MEDICINA DE CAMPOS/FUNDAÇÃO BENEDITO PEREIRA NUNES

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise histopatológica em placentas e pele de pacientes infectados com Zika e de tecidos de natimortos originário de casos de microcefalia

Pesquisador: Marciano Viana Paes

Área Temática:

Versão: 1

CAAE: 65924217.4.3001.5244

Instituição Proponente: FUNDACAO OSWALDO CRUZ

Patrocinador Principal: FUN CARLOS CHAGAS F. DE AMPARO A PESQUISA DO ESTADO DO RIO DE JANEIRO - FAPERJ

DADOS DO PARECER

Número do Parecer: 2.091.607

Apresentação do Projeto:

Trata-se de projeto envolvendo instituições co-participantes, que já foi avaliado pelo CEP mais próximo à Instituição proponente principal. Este CEP foi elencado para avaliação em função da participação da Instituição Hospital Plantadores de Cana e UTI neonatal Ceplin, ambos nesta cidade, como locais de obtenção de amostras. Projeto apresenta delineamento adequado e capaz de responder os objetivos da pesquisa .

Objetivo da Pesquisa:

Objetivo claro e preciso que se insere adequadamente no desenvolvimento do projeto de pesquisa.

Avaliação dos Riscos e Benefícios:

Riscos: São descritos os riscos quanto ao procedimento da biópsia cutânea:- Pequeno risco de sangramento ou infecção no local da biópsia.- Reação de hipersensibilidade (alergia) ao anestésico usado. Ocorrência considerada extremamente rara.- Prurido no local da biópsia, de ocorrência variável, em geral após 5 a 7 dias do procedimento. Resolve-se com a retirada dos pontos.- Pequena cicatriz no local da biópsia, que pode ser imperceptível, na dependência do processo de reparo cicatricial inerente a cada indivíduo. Com o material cirúrgico usado o tamanho médio da cicatriz é de 5mm.