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Protective Role of Palmitoleate against Zika Virus-Induced Apoptosis in Placental Trophoblasts

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PROTECTIVE ROLE OF PALMITOLEATE AGAINST ZIKA VIRUS-INDUCED APOPTOSIS IN PLACENTAL TROPHOBLASTS

by

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Zika virus (ZIKV) infection in pregnant women causes congenital Zika syndrome which involves birth defects such as intrauterine growth restriction (IUGR), retinal defects and microcephaly in the fetus. ZIKV infection results in placental pathology which plays a crucial role in disease transmission from mother to fetus. Currently, there is no Food and Drug Administration (FDA) approved vaccine or therapeutic drug against ZIKV. In this thesis, we have elucidated the cell signaling pathways connected to ZIKV-induced apoptosis in trophoblasts and also the protective effect of palmitoleate against ZIKV-induced apoptosis. First, we demonstrated the molecular mechanism behind ZIKV-induced apoptosis using an *in vitro* model with JEG-3, JAR and HTR-8 cells. We found that persistent endoplasmic reticulum stress (ER stress) causes activation of c-Jun N-terminal kinase (JNK), a stress kinase that leads to trophoblast apoptosis. Next, we studied the protective role of the nutrient compound palmitoleate, an omega-7 monounsaturated fatty acid, in ZIKV infected trophoblasts. Palmitoleate has been shown to have lipokine activity and significant positive effects on metabolism. Palmitoleate treatment post-infection showed a significant decrease in the percentage of apoptotic nuclei and caspase 3/7 activity when compared to ZIKV-infected cells without palmitoleate treatment. Moreover, quantification of viral RNA revealed that there was a

notable decrease in viral load in palmitoleate-treated cells. To substantiate the protective role of palmitoleate against ZIKV-induced apoptosis, we treated trophoblasts with palmitate, a 16-carbon saturated fatty acid. Interestingly, we observed that palmitate did not show any protection against ZIKV-induced ER stress and apoptosis. In contrast, palmitoleate treatment enhanced survivability coupled with a non-significant reduction in plaque-forming units/mL of cell culture supernatant. Further, we observed high lipid droplet accumulation along with a significant reduction in IL-1β expression with palmitoleate treatment in infected cells. In conclusion, palmitoleate treatment is protective against ZIKV-induced ER stress and apoptosis in trophoblasts. Palmitoleate can be used as a nutrient compound to prevent placental pathology in ZIKV infected pregnant women and adverse birth defects in the fetus; nevertheless, further studies are necessary.

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Dedicated to my parents

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LIST OF ABBREVIATION

Ab – antibody

ADE - Antibody-Dependent Enhancement

ADRP- Adipose Differentiation- Related Protein

Akt-mTOR - Protein Kinase B- Mammalian Target of Rapamycin

AMPK - Adenosine Monophosphate-Activated Protein Kinase

ANOVA - Analysis of Variance

ASK1 - Apoptosis Signal-Regulating Kinase 1

ANXA1 - Anti-Inflammatory Protein Annexin 1

ATCC - American Type Culture Collection

ATF-6 - Activating Transcription Factor 6

ATGL - Adipose Triglyceride Lipase

bax - BCL2 Associated X

Bcl2 - B-cell lymphoma 2

Bip - Immunoglobulin binding protein

BSA - Bovine Serum albumin

bp - Base pairs

C – Celcius

CD – Cluster of Differentiation

cDNA - Complementary DNA

CHAC1- ChaC Glutathione-Specific gamma-glutamylcyclotransferase 1

CHOP - CCAAT/Enhancer-Binding Protein (C/EBP) Homologous Protein

CZS - Congenital Zika Syndrome

DAPI - 4′,6-Diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle Medium

DNA- Deoxyribonucleic Acid

DTT – Dithiothreitol

EDEM-1 - ER Degradation-Enhancing α-Mannosidase-like 1

EDTA - Ethylenediaminetetraacetic acid

E protein - Envelope protein

eIF2α - Eukaryotic Initiation Factor 2 alpha

ER - Endoplasmic Reticulum

ERAD – Endoplasmic Reticulum-Associated Protein Degradation

ERO1α - Endoplasmic Reticulum Oxidoreductin alpha

FAM134B - Family with Sequence Similarity 134 Member B

FBS - Fetal Bovine Serum

FcRn - Neonatal Fc Gamma Receptor

FDA – Food and Drug Administration

g - Relative centrifuge force

GADD45 - Growth Arrest and DNA Damage-inducible 45

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GRP78 - 78-kDa Glucose-Regulated Protein

GBS - Guillain-Barré Syndrome

h - hour

HDAC1- Histone deacetylase 1

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HERPUD1 - Homocysteine Inducible ER Protein with Ubiquitin Like Domain 1

Hpi - hours Post Infection

HSL - Hormone-Sensitive Lipase

HRP-conjugate - Horseradish peroxidase conjugate

HUVEC - Human Umbilical Vein Endothelial Cells

IFNAR 1 - Interferon α/β receptor 1

IFN-γ – Interferon-gamma

IGF2 - Insulin-like Growth Factor II

IL-6 - Interleukin

IRE1α - Inositol Requiring Enzyme-1

IUGR - Intrauterine Growth Restriction

JNK - c-Jun N-terminal Kinase

Kb – kilobase

KCl - Potassium chloride

LDL - Low-Density Lipoprotein

kDa - kilo Dalton

MAPK - Mitogen-Activated Protein Kinase

MEM - Minimum Essential Medium

mM - milli molar

min - minute

mL – milliliter

mRNA - Messenger RNA

MOI - Multiplicity of Infection

MRV- MR-766 ZIKV strain

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

n - Sample size

Na3Vo4 - Sodium orthovanadate

NaF- Sodium fluoride

NaF- Sodium fluoride

NaCl - Sodium chloride

nm – nano meter

NS1- Nonstructural protein 1

PA – Palmitate

pfu - plaque-forming units

PO – Palmitoleate

PBS - Phosphate-buffered Saline

PERK- Protein kinase RNA-like Endoplasmic Reticulum kinase

PMSF - Phenylmethylsulfonyl fluoride

p-value - probability value

PPARα - Peroxisome Proliferator-Activated Receptor alpha

PRV- PRVABC-59 ZIKV strain

p – phosphorylation

qPCR - Quantitative Polymerase Chain Reaction

RAG-1 - Recombination Activating Gene-1

RANTES - Regulated on Activation, Normal T cell Expressed and Secreted

RBC - Red Blood Cell

RE site - Restriction Enzyme site

RIPK1/RIPK3 - Receptor-Interacting Serine/Threonine-Protein Kinase 1/3

RLR - RIG-I (Retinoic acid-inducible gene I) like receptor

RNA - Ribonucleic Acid

r-MRV - Recombinant MR ZIKV

rpm - rotations per minute

rRNA - Ribosomal RNA

SCD1 - Stearoyl Coenzyme A Desaturase 1

SDS-PAGE - Sodium Dodecyl Sulphate- Polacrylamide Gel Electrophoresis

SEM - Standard Error of the Mean

TBS - Tris-Buffered Saline

TBST - Tris-Buffered Saline, 0.1% Tween 20

TIM1 - T-cell Immunoglobulin and Mucin domain 1

TLR-3 - Toll-Like Receptor-3

TNF-α – Tumor Necrosing Fator alpha

TRAF2 - TNF Receptor-Associated Factor 2

TRB3 – Tribbles 3

UPR - Unfolded Protein Response

VEGFR-2 - Vascular Endothelial Growth Factor Receptor-2

Veh –Vehicle

WHO - World Health Organization

XBP1- X-box Binding Protein 1

ZDBP1 - Z-DNA-Binding Protein 1

ZIKV - Zika Virus

Z-VAD-FMK - Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Zika virus (ZIKV) is a *Flavivirus* from the *Flaviviridae* family (1). ZIKV is a positive-sense RNA virus belonging to group IV in the Baltimore system of virus classification (2). It is an arbovirus (vector-borne virus) closely related to viruses like Dengue virus, yellow fever virus, West Nile virus, Japanese encephalitis virus, tick-borne encephalitis virus and chikungunya virus (2-4). ZIKV infection can be compared to pathogens such as toxoplasmosis, Rubella, Cytomegalovirus, and Herpes simplex viruses type 1 and 2, all of which cause a mild infection in the mother and is vertically transmitted to the developing fetus, causing congenital anomalies (5).

ZIKV was first isolated from a sentinel monkey kept for studying mosquito-borne diseases, and it was also later isolated from *Aedes africanus* mosquitoes, confirming its vector-borne transmission in the Zika Forest of Uganda (6). Intracerebral inoculations of ZIKV in young mice showed extensive neurological lesions, while inoculation from the mice to non-human primates resulted in a self-limiting febrile condition in a few members. Later, neutralizing antibodies were found in both humans and monkeys on serological screenings (7). The first human case was reported during the isolation process of the virus, wherein the clinical signs were described as pyrexia along with rashes by Simpson in 1964 (8). The disease was relatively insignificant with sporadic outbreaks in the Asian and African continents till 2006 (9).

Zika virus (ZIKV) was considered a public health emergency by World Health Organization (WHO) in 2015 after the disease started to have a widespread outbreak across different continents around the globe (10). The occurrence of neurological abnormalities, especially in newborn infants, created a lot of concern regarding this disease outbreak (11).

1.2 ZIKV epidemiology

Until April 2007, there were very few cases of ZIKV infection reported in humans(12). The first widespread cluster of ZIKV outbreaks was reported from Yap island in Micronesia (13). Around 2013-2014, another outbreak with a considerable number of infections occurred in French Polynesia (14). Reports of various modes of transmission other than mosquitoes and involvement of neurological disorders such as Guillain-Barre syndrome in a subset of the population were observed during this outbreak (15,16). Later from 2014 onwards, places such as New Caledonia, Cook Islands, Easter Island, Anuatu, Solomon Islands, Samoa, and Fiji experienced ZIKV outbreaks (16). The presence of vectors and travel-related introductions of ZIKV to a population without any prior exposure, along with other existing arboviral infections like dengue and Chikungunya, may have favored the transmission of disease at a higher altitude (17-20). In 2015 ZIKV had spread to Brazil, and several hypotheses suggested that the spread could be due to travel associated with big events such as the soccer World Cup and other tournaments (21). Later, ZIKV spread to other parts of the American continent including Colombia, Honduras, Puerto Rico, the Dominican Republic, Jamaica, and Haiti (22). In the mainland of the United States of America (USA), cases were reported in the state of

Florida in 2016 (23). In Asia, outbreaks were seen in Vietnam, Singapore, Philippines and Thailand (24). As the ZIKV cases began to decline the public health emergency declared by WHO was brought to an end in late 2016 (25).

1.3 ZIKV Strains

Genetic changes in the ZIKV, involving complex interactions between the vector, human populations and non-human primate populations led to the evolution of the virus (26). Two lineages of ZIKV are 1)Asian origin and 2)African origin. The African strain has two groups, the Ugandan versus the Nigerian group. The strain originally isolated from Rhesus macaque in the Zika forest is MR-766. Whereas IbH is the first strain isolated from the human blood in Nigeria (27). The first isolated Asian ZIKV strain is from Malaysia with the prototype strain P6-740, and the cluster includes strains from Cambodia, French Polynesia and other Asian countries. In contrast, some reports describe that the African strain lyses the placental cells more effectively than Asian strains but has the same replicative efficiency (28).

ZIKV strains in the American continent that circulated from the 2015 Brazil outbreak, evolved from the Asian lineage (29). Travel-related to major sports events have mainly contributed to the spread of the virus from Pacific islands including French Polynesia to Brazil (30). The presence of a new glycosylation motif in an asparagine residue at position 154 of envelope protein in the 2007 Yap strain- EC Yap and the French Polynesian H/PF/2013 strain could explain the gain in their virulence when compared to MR766 which does not have this glycosylation motif (31).

1.4 Transmission of ZIKV

Usually, the disease is spread by the bite of the infected mosquito (*Aedes aegypti, Aedes albopictus*)(32). Aedes mosquito larvae and pupae can get infected in sewage or water contaminated with urine from an infected person with low ZIKV concentrations(33). The infection can be vertically transmitted from infected mother to fetus. It can also be sexually transmitted, as ZIKV RNA is detected in semen samples of infected patients (34,35). Although around 3% of the total ZIKV cases account for sexually transmitted cases, a study suggests that semen suppresses the binding of ZIKV to cells(36). Blood transfusions from infected individuals could also be a potential source of infection. (37). The virus replicates in the epithelial cells of the mosquitoes' gut and later spreads to the mosquitoes' salivary gland: then, the virus spreads to humans during a mosquito bite (38,39). The receptors in the dermal fibroblasts, immature dendritic cells and keratinocytes facilitate viral entry and support viral replication (40). Wild macaques are naturally susceptible to ZIKV infection (41). The arbovirus infection follows a sylvatic cycle with non-human primates as the reservoir of the virus (42). They serve as the connecting bridge for ZIKV circulation among the mosquitoes and transmission to humans due to the extensive urbanization in the present-day scenario (43).

1.5 ZIKV structure

ZIKV is icosahedral in symmetry, ~40 nm with a nucleocapsid ~25-30 nm and surface projections ~5-10 nm (44,45). Its genome is 10.8Kb with 5' NCR (translation via a methylated nucleotide cap or a genome-linked protein) and 3' NCR (translation, RNA packaging, cyclization, genome stabilization, and recognition)(46-48). The virion consists of an envelope (E protein) covering the majority of virion surface, non-structural proteins

NS1 for virion production, NS3, and NS5are large, highly-conserved proteins, NS2A, NS2B, NS4A, and NS4B are small, hydrophobic proteins and NS4B, NS5 are targets for evolution(48-50). Functions of individual ZIKV proteins are enlisted in the table below.

Table 1.1 ZIKV viral proteins

Protein	Function
Envelope	Host cell binding and membrane fusion (51)
Capsid	Viral protein surrounds nucleic acid (52)
Membrane protein	Proteolytic cleavage of a pre membrane protein from membrane protein in the Golgi apparatus results in the release of the virus (53)
NS1	RNA replication (54)
NS2A	Modulates different components of the virus during assembly (55)
NS2B	Cofactor of NS3 protease (56)
NS3	Protease and helicase domain for polyprotein possessing & nucleoside triphosphtase (NTPase)/ RNA triphosphatase (RTPase) activities (56)
NS4A	Evasion of the innate immune response, associated with replication complex (57,58)
NS4B	Evasion of the innate immune response (59)
NS5	Methyl transferase (MTase) and RNA dependent RNA polymerase (RdRp)(60)

1.6 ZIKV replication

Virus entry into the cell occurs by the initial recognition of host receptors by glycosylated regions on the envelope protein of the ZIKV (61). Endocytosis of the infectious viral particle occurs by clathrin-coated vesicles. A low pH environment within the endosome facilitates conformational changes in the envelope protein of the virus, resulting in fusion to the endosome and thereby releasing the positive-strand RNA of the virus (62). The positive-strand gets translated in the endoplasmic reticulum of the host cells into a polyprotein that gets cleaved by the host cell proteases and the viral non-structural proteins such as NS3 and NS2B which is a co-factor for protease. Non-structural proteins NS5 (RNA-dependent RNA polymerase), NS3 (helicase) also replicate the positive-sense RNA strand to form a negative-sense RNA strand(63). The negative-sense RNA strand serves as a template for further production of a new positive sense RNA strand. The newly produced positive sense RNA strand can either be translated or further used for viral genome replication (64). After the assembly of structural proteins around the viral genome, they are translocated to the Golgi apparatus where they become mature virion by cleavage of the precursor membrane protein and exit the host cell (65).

1.7 Clinical findings and Congenital Zika Syndrome

In normal healthy children and adults, ZIKV infection usually presents a mild febrile disease with rashes and joint pain (3). Other clinical signs may include diarrhea, cough, lymphadenopathy and photophobia (66). Pregnant women typically develop symptoms like rashes during ZIKV infection (67). ZIKV infection in pregnant women results in both congenital brain and ocular defects in the fetus. Brain defects include microcephaly, cerebral atrophy, subcortical calcifications, agyria, hydrocephalus and ventriculomegaly

(68). Ocular defects include microphthalmia, optic nerve defects, cataract and intraocular calcifications. Congenital contractures, reduced musculoskeletal movements, dysphagia, hypertonia, hypotonia, seizures and irritability are also reported in infants with *in utero* ZIKV infection (69). Further, a case-control study showed that women with ZIKV infection during the early stages of pregnancy were more likely to have babies with congenital Zika syndrome (CZS) (70). ZIKV infection is also associated with the development of Guillain-Barre syndrome in some adults which is an autoimmune condition affecting the nervous system (71).

1.8 Diagnosis, treatment and prevention of ZIKV infection

In suspected ZIKV cases, a diagnosis is usually based on laboratory confirmation using IgM detecting serological test or RT-PCR based on E and NS2B genes (72-74). In a particular place when there are ongoing outbreaks, it is recommended for pregnant women to get tested for ZIKV infection (75). Serology tests can detect ZIKV as early as one week after suspected infection, but cross-reacting antibodies from other Flaviviruses can result in false-positive serological results (76). RT-PCR can be used to detect the disease during initial viremia in urine samples, cord blood and placental samples (77).

Currently, there is no approved vaccine for the effective prevention of the disease (78). Only supportive treatment is available if infected(79). Implementing effective mosquito control strategies in places with ZIKV infection is crucial to break the chain of ongoing disease spread (80). Avoiding travel to areas with ongoing ZIKV outbreaks, especially if pregnant or planning to get pregnant are some of the ways to reduce the risk of infection (81). The option of using genetically modified *Aedes aegypti* mosquitoes to reduce the

population of wild type mosquitoes to control mosquito-borne disease exists yet it is considered an emerging risk (82)

1.9 ZIKV vaccines and drug development

ZIKV vaccine development is challenged by the target audience; it must be safe for pregnant women, to prevent neurological disorders in adults and fetuses (83,84). Despite the challenges, several vaccine candidates have entered preclinical animal studies and phase I clinical trials. Some of the vaccine candidates which have entered phase I clinical trials that are noteworthy to mention include DNA vaccines by Inovio Pharmaceuticals and NIH, whole purified inactivated vaccine by WRAIR/Sanofi Pasteur Limited and Live, Dengue virus vectored vaccine by Butantan Institute (83). Another major issue in vaccine development and translation of the vaccine technology into use is that ZIKV outbreaks had waned, making it too challenging to test the effectiveness of the vaccine without ongoing active disease transmission, as well as the slow decline in funding, supports aiding in vaccine development (85). Several drug repurposing studies have been conducted and found to be effective against ZIKV infection. However, there are no FDA-approved drugs available for ZIKV infection because most of the drugs don’t have enough data to support safety in pregnant women (85). Few examples of already existing drugs with anti-ZIKV activity are suramin, nitazoxanide, chloroquine (anti-protozoal drugs), niclosamide, ivermectin (anthelmintics), mycophenolic acid (an immunosuppressant drug), PHA-690509 (cyclin-dependent kinase inhibitor) and sofosbuvir (an anti-viral drug effective against hepatitis C virus, (86). Sofosbuvir has shown promising results in preventing ZIKV transmission from mother to fetus in pregnant mice and pregnant non-human primate models (87,88).

1.10 Nutraceuticals against ZIKV infection

Nutraceuticals are naturally occurring compounds in food with health or medicinal value (89). In an *insilco* analysis, around 2263 plant-derived compounds were screened and 43 of those compounds had anti-viral potential against ZIKV. Some of the well-known plant-derived compounds which could bind to ZIKV proteins are kanzonol V from licorice root (*Glycyrrhiza glabra*), cinnamoylechinaxanthol from *Echinacea* root; cimiphenol from black cohosh (*Cimicifuga racemosa*), rosemarinic acid from rosemary (*Rosmarinus officinalis*), lemon balm (*Melissa officinalis*), and common sage (*Salvia officinalis*) (90). Isoquercitrin, which is a flavonoid compound, has been found to interfere with the entry of the virion into the target cells (91). Curcumin, a bioactive compound in turmeric also prevents ZIKV attachment to cells (92). Gossypol, a phenolic compound seen in cotton seeds, has anti-ZIKV activity by interacting with the envelope protein domain III of the virus (93). F-6 and FAc-2 fractions abundant with cyclic diterpenes with aldehyde groups from *Dictyota menstrualis*, a brown seaweed in Brazil, have potent anti-viral activity against ZIKV (94). Polyphenols such as delphinidin and epigallocatechin gallate, which are available in natural products such as wine and tea, exhibited antiviral activity against ZIKV in an *in-vitro* model (95). Berberine, an isoquinoline alkaloid seen in *Berberis vulgaris, as well as* Emodin, an anthraquinone derivative available in *Rheum palmatum*, *Polygonum multiflorum*, *Aloe vera*, and *Cassia obtusifolia* were found to have anti-viral activity against ZIKV (96). A flavonoid compound called naringenin seen in citrus plants exhibits anti-ZIKV activity by binding to the protease domain of the NS2B-NS3 protein (97). 6-deoxyglucose-diphyllin, seen in *Justicia gendarussa* could prevent the facilitation of an acidic environment within the

lysosome or endosome that allows the virus to fuse. Further, 6-deoxyglucose-diphyllin also was protective against ZIKV infection both in cell culture as well as in an immunocompromised mice model (98). Hippeastrine hydrobromide seen in *Lycoris radiate* was found to be protective against the neuronal damage caused by ZIKV along with having other anti-viral activity (99). *Doratoxylon apetalum* plant extract, which is already known to have a protective role against oxidative stress in cells, also had anti-viral activity by preventing ZIKV entry into the cells (100). 25-hydroxy cholesterol seen naturally in the hosts was found to have anti-viral activity and was able to prevent ZIKV associated clinical signs in both mice and macaque models. Similarly, 25-hydroxy cholesterol also inhibited ZIKV infection in human corticoid organs and microcephaly in newborn mice pups (101). Omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) was found to have a protective effect against ZIKV-induced neuronal damage in a cell culture model (102). Nutraceuticals investigated for anti-ZIKV activity enlisted in the table below:

Table 1.2 Natural compounds effective against ZIKV infection

Natural compounds with anti-ZIKV activity	Reference
Isoquercitrin	(91)
Curcumin	(92)
Gossypol	(93)
F-6 and FAc-2 fractions from *Dictyota menstrualis*	(94)

Polyphenols - delphinidin and epigallocatechin gallate	(103)
Berberine	(104)
Naringenin- flavonoid	(97)
6-deoxyglucose-diphyllin	(98)
Hippeastrine hydrobromide	(99)
***Doratoxylon apetalum* plant extract**	(100)
25-hydroxy cholesterol	(101)
DHA	(102)

1.11 Nutrition and ZIKV

The nutritional status of the host can contribute to the evolution of the viral disease by mutations contributing to virulence (105). Likewise, the nutritional status of the host can also play a role in the vector-borne disease evolution (106). Evidence of supportive nutritional therapy has been important in arboviral infections like dengue, but there are very limited reports surrounding the topic of nutritional parameters in the context of ZIKV infection (107). There is an interesting hypothesis which states that the neuronal damage associated with ZIKV could result from the retinoid compounds that have leaked from the liver tissue during ZIKV infection (108). A study found an association between nutrition and motor function in children with palpsy; this could also possibly affect the outcomes in infants affected with ZIKV infection (109). A correlation between ZIKV infection and anemia has been seen in several cases, and in contrast, other reports show no evidence of anemia with ZIKV infection (110,111). A study using a mice model revealed that protein malnutrition could be a risk factor to develop congenital Zika

syndrome. The results of this study are possibly correlated to the fact that undernutrition is commonly seen in regions with major Zika outbreaks such as Brazil (112). Another interesting entomology study found that blood meals containing ZIKV showed prominent infection in mosquitoes when compared to protein meals containing ZIKV fed mosquitoes (113). A study showed that folic acid supplementation reduced ZIKV infection in a cell culture model associated with the placental barrier and showed improved postnatal outcomes in fetuses from ZIKV infected pregnant mice (114). Therefore, monitoring folic acid nutrient status in ZIKV prone endemic areas and enabling its supplementation could help to ameliorate the adverse effect on the fetus observed during ZIKV infection (114).

1.12 Immunological response to ZIKV infection

ZIKV can evade the innate immune responses generated by the host via suppression of type I interferon and the subsequent activation of interferon-stimulated genes via non-structural viral proteins like NS5 and NS4A (57,115). Interferon λ is known to protect against ZIKV infection in females in cases of the sexual route of transmission (116). A recent study shows that at least a 12-month interval between an initial Dengue virus infection confers an effective cellular immune response against a subsequent ZIKV infection (117). There are also contrasting studies that report pre-existing antibodies against the Dengue virus aggravating ZIKV infection (118,119). Production of IgM, which is the first antibody to appear against the first encounter with a pathogen, is found to be affected in populations with pre-existing antibodies against flavivirus (120). Pre-existing antibodies against ZIKV can also cause severe outcomes after subsequent dengue infection due to antibody-dependent enhancement (ADE) (121,122).

Studies have shown that interferon λ could be protective against ZIKV infection in the placenta (123-125). On the other end, a recent study has shown that placental alkaline phosphatase stabilized by binding immunoglobulin protein (Bip) aids ZIKV infection in placental cells (126). ZIKV activates an inflammatory response following infection by inflammasome complex formation resulting in IL-1β release (127-129). The placenta undergoes extensive inflammatory changes following ZIKV infection with upregulation of cytokines like IFN-γ and TNF-α and chemokines like RANTES (regulated on activation, normal T cell expressed and secreted) and VEGFR-2 (vascular endothelial growth factor receptor-2) (130,131). Toll-like receptor-3 (TLR-3), which is a pattern recognition receptor of the innate immune system that senses double-stranded RNA which is seen during ZIKV replication, is involved in activation of the inflammatory response particularly in astrocytes (132). Similarly, babies with congenital Zika syndrome were found to be highly associated with single nuclear polymorphisms in TLR-3 or TNF-α (tumor necrosis factor-alpha) alleles (133). These studies allude to the importance of the inflammatory response in the ZIKV-induced pathological manifestations in the host. Another important component that protects against ZIKV infection is a cell-mediated immune response and its associated neutralizing antibody generation which is crucial for immunity. Meanwhile, immunologically privileged parts of the body like the gravid uterus might be vulnerable to ZIKV infection (134). A sero-surveillance report in Fiji and French Polynesia of Pacific Islands that experienced ZIKV outbreaks in 2013-2014, currently displays a pattern wherein the younger population comprising of children still have neutralizing antibodies against ZIKV but the older population comprising of adults showed a decline in the neutralizing antibody titer (135).

ZIKV and inflammation: ZIKV is known to affect various organs in the body from the eyes to the reproductive organs. ZIKV especially strains from the Asian lineage, are known to produce an inflammatory response in the body (136). A study in chicken embryo livers showed that ZIKV from Asian lineage (isolated from China) elicited a very intense inflammatory response in comparison to dengue virus infection (137). ZIKV infection in non-pregnant mice caused acute inflammation of the ovaries without any long-term effects on the overall reproducing ability (138). An immunocompetent C57BL/6J mice model with intravenous challenge study also suggests that ZIKV induces an inflammatory environment in the blood-brain barrier. Additionally, ZIKV elicits a strong inflammatory response in human retinal epithelial cells, chemokine, CXCL10 was highly expressed following infection (139). There is also evidence of placentitis in pregnant women following ZIKV infection (131,140). Transcriptome analysis of human umbilical vein endothelial cells (HUVEC) showed upregulation of several cytokines, chemokines and matrix metalloproteinases on ZIKV infection (141). There are also cases of ZIKV-induced meningitis, encephalitis and myelitis in certain patients (142). In a recent study, IL-22 was attributed to inflammation of the brain in newborn mice pups infected with ZIKV(143). Mice models suggest that ZIKV can cause orchitis and epididymitis via pro-inflammatory cytokines and chemokines (144). Overall, ZIKV infection elicits an inflammatory response in the host.

1.13 Cell death in ZIKV infection

ZIKV is known to initiate apoptosis, in which both intrinsic pathways and extrinsic pathways contribute to cell death in the neuronal progenitor cells, leading to microcephaly (145,146). The extrinsic pathway of apoptosis is activated by cytokines and

death ligands like FasL while the activation of the intrinsic pathway is by cytochrome C released from damaged mitochondria. Both intrinsic and extrinsic pathways merge into a common pathway by activating effector caspases that triggers apoptosis also known as programmed cell death (147). Also, activation of the necroptotic pathway via RIPK1/RIPK3 (Receptor-interacting serine/threonine-protein kinase 1/3) and Z-DNA-binding protein 1 (ZBP1) favors succinate dehydrogenase formation in neuronal cells which interfere with ZIKV replication was reported (148). Pyroptosis is also known to be associated with ZIKV infection by its activation of NLRP3 complex formation, which initiates caspase-1 activation (149,150). A recent study highlights the mechanism of pyroptosis in neuronal progenitor cells following ZIKV infection (151). ZIKV replicates, forming complexes inside the endoplasmic reticulum, resulting in large vacuoles in the cytoplasmic compartment of the cell and leads to paraptosis in human epithelial cell lines, human primary fibroblasts and astrocytes (152). Necrotic cell death involves swelling of internal cellular organelles, which eventually rupture and are released outside the cell (153). Necrotic lesions involving the brain are also observed in animal models including immunocompetent mice and non-human primates (154,155). Autophagy, once considered as a cell survival pathway by recycling cellular cargoes via lysosomes to build new cellular components, under certain conditions can activate cell death directly or indirectly (156). ZIKV causes extensive activation of autophagy by downregulating Akt-mTOR (Protein kinase B- Mammalian target of rapamycin) signaling pathways aiding in replication (157). In contrast, another study showed that activation of the mTOR pathway inhibits autophagy and facilitates ZIKV replication (158). Together, ZIKV infection

induces apoptosis, necrosis, pyroptosis, paraptosis and autophagy-dependent cell death pathways.

1.14 ZIKV and placenta

Structure and function of the placenta: The placenta is a temporary organ that develops between the fetus and the mother and participates in nutrient transport, waste exchange and metabolism (159). In humans, the fetal part of the placenta is composed of the placental disc, umbilical cord, amnion and chorion, whereas the maternal part from the endometrium of the uterus is the decidua (160). The major cell type that predominates in the placenta is the trophoblasts, which include syncytiotrophoblasts, villous cytotrophoblasts, and extravillous trophoblasts that are characterized by a highly invasive nature, supported by the maternal decidual cells (161). The placental functional units are called villi, formed by an outer layer of trophoblasts with a stromal core (162). The placental villi participate in nutrient absorption for the growing fetus like the intestinal villi that absorb nutrients from digested food in the gastrointestinal tract (163,164). Cytotrophoblasts are a layer of cells that cover the stromal core located between the basement membrane and syncytiotrophoblasts (165). Extravillous trophoblasts are cells that migrate from the villi and are involved in uterine remodeling by forming trophoblast cell columns. Syncytiotrophoblasts are multinucleated cells covering the entire placental units, 2-3 cytotrophoblasts fuse to form syncytiotrophoblasts (166,167). Both syncytiotrophoblasts and extravillous trophoblasts are differentiated from the cytotrophoblasts (168). The stromal core of the placenta is richly supplied with blood vessels that originate from the mesenchymal stem cells and Hofbauer cells (placental macrophages)(166).

ZIKV infection in the placenta and its consequences: ZIKV has been demonstrated to replicate in the human placenta, including the Hofbauer cells and trophoblasts (169-172). T cell immunoglobulin and mucin domain 1 (TIM1), Tyro3 and Axl (tyrosine-protein kinase receptors) are considered the cofactors for viral entry into cells. There is a considerable expression of TIM1 in cytotrophoblasts, fibroblasts, umbilical vein endothelial cells, Hofbauer cells and amniochorionic membranes of the placenta, whereas Tyro3 and Axl are variably expressed in these cells (173). The first trimester of pregnancy was reported to be most susceptible to ZIKV infection (70,140,174,175). While some reports demonstrated that congenital Zika virus syndrome was also observed with ZIKV infection during the second and the third trimesters of pregnancy (176-178). Placental enlargement is an early clinical feature noticed in ZIKV infected pregnancies(179). Pregnant women who delivered babies with microcephaly typically exhibit clinical signs of ZIKV infection around the start of mid-gestation (8-16 weeks); this is when maternal blood circulation is well established via the placenta (180). Breaches in the placental barrier could be detected in placental sections from ZIKV infected women (181). A study using a cell culture model suggests that ZIKV can breach the placental barrier by disruption of tight junctions between the cells of the placenta. Further, ZIKV virions take a transcytosis route to enter the tightly regulated placental barrier and blood-brain barrier (182). Another study using placenta samples from ZIKV infected women infected reported that there are ongoing changes in the tight junctions of the syncytiotrophoblasts with the decrease in the claudin 4 expression that leads to potential breaches of the placental barrier (183). ZIKV could also be transferred from the placenta to the fetus utilizing secretory autophagy (184).

ZIKV infection alters the lipid metabolism of placental cells by favoring lipid droplet deposition, which contributes to the ongoing inflammatory process coupled with mitochondrial dysfunction (131). Another study reported the association of sphingolipids and deposition of ceramides with ZIKV replication particularly in neuronal progenitor cells (185). A study in ZIKV infected women showed that the placental samples had an inflammatory state even without the actual presence of ZIKV virion and also connects this to the involvement of a modulatory role of anti-inflammatory protein annexin 1 (ANXA1) as a result of ZIKV exposure to the placenta (186). Further, the presence of non-neutralizing flavivirus antibodies was also shown to facilitate or enhance viral infection and spread to syncytiotrophoblasts via neonatal Fc gamma receptor (FcRn) (187). A Recombination-activating gene-1(RAG-1) knockout mice treated with interferon α/β receptor (IFNAR 1) antibody study shows that neutrophils and macrophages of the dam can play an important role in limiting ZIKV spread to the fetus (188). A recent study in twins found that trophoblasts from the baby without the congenital Zika syndrome had a differential activation of genes which contributed to its ability to mount a better immune response against the infection (189). Expression of Insulin-like growth factor II (IGF2), which is necessary for the proper development of the baby, was found to be inhibited in placental samples from ZIKV infected women (190). Researchers were even able to rescue ZIKV in vitro from mesenchymal stem cells derived from the placenta of a woman who had cleared the infection and delivered a baby negative for ZIKV infection alluding to ZIKV persistence (191).

In a normal pregnancy, monocytes are polarized to the M2 state to be compatible with the placenta and uterine environment and generate an anti-inflammatory or

immunosuppressive state with potential suppression of type I interferon response. But monocytes are predominantly polarized to the M1 pro-inflammatory phenotype, resulting in adverse outcomes of pregnancy due to ZIKV African strain infection (196). The $CD14^{+}CD16^{+}$ monocytes in the peripheral circulation are also affected by ZIKV infection, producing a 100-fold increase in the expression of CXCL12 and IL-6 in ZIKV infected women (195). Further, the presence of non-neutralizing flavivirus antibodies was also shown to facilitate or enhance viral infection and spread to syncytiotrophoblasts via neonatal Fc gamma receptor (FcRn) (187).

Animal models of ZIKV infection during pregnancy: A study of ZIKV infection in pregnant rhesus macaques confirmed extensive uteroplacental pathology leading to decreased oxygen permeability. This model recapitulates the adverse outcomes noticed in humans such as reduced intrauterine growth, and still-birth (192). Similarly, immunocompetent wild-type mice mated with immunocompromised (interferon α/β receptor knock-out (*IFNAR* -/-)) resulted in heterozygous pups which are immunocompetent exhibited extensive placental pathology and fetal damage following ZIKV infection (193,194). Further extensive placental labyrinth apoptosis contributing to hypoxia in the heterozygous fetus resulting in early resorption (194).

1.15 Endoplasmic reticulum (ER) stress in ZIKV infection

The ER is an important subcellular organelle in a eukaryotic cell wherein the oxidizing environment and chaperones serve the purpose of protein folding, and it also serves as the site for steroid hormone and lipid synthesis (197). Several conditions including hypoxia, nutrient deprivation and perturbations in the calcium homeostasis cause accumulation of

misfolded proteins in the ER leading to ER stress. This in turn results in the activation of several interactive signaling pathways known as the unfolded protein response (UPR). During ER stress, cells can undergo two possible fates: cell survival and cell death. The UPR tries to remove the misfolded proteins in the ER using different pathways to help cell survival, but persistent or prolonged ER stress can overwhelm the protective mechanism that aids in cell survival, resulting in the activation of cell death pathways like apoptosis (198).

There are 3 main arms of the UPR activation in cells with ER stress as shown in Figure1.1. Usually, these sensors of unfolded proteins are at an inactive monomeric state with the association of Bip. It dissociates with a loss of homeostasis between folded and unfolded proteins leading to activation of ER stress sensors: 1) protein kinase RNA like endoplasmic reticulum kinase (PERK), 2) inositol requiring enzyme 1 alpha (IRE1α) and 3) activating transcription factor 6 (ATF6).

Flaviviruses are closely associated with ER stress as they replicate within the cellular membrane-bound organelles especially the ER. Accumulation of structural and non-structural proteins in the ER results in the formation of convoluted spherules which activates UPR. Apart from this, ZIKV also remodels the ER in terms of its protein and lipid content (199,200). The shift from direct to indirect neurogenesis is done by decreasing UPR activity. Any dysregulation in this response could lead to microcephaly. ZIKV infection in neuronal progenitor cells activates PERK and IRE1α signaling pathways, suggesting the molecular mechanism behind the cause of microcephaly (201). Further, ZIKV could indirectly activate UPR in response to ER stress by inducing cytokine and chemokine production in infected and non-infected neuronal cells. This also

attracts the resident macrophages (microglial cells), amplifying the response (202). ZIKV can also thwart the UPR mechanism by cells to counteract ER stress by downregulating Bip in A549 cells (human alveolar basal epithelial cell line) (203).

Additionally, there is cross-talk between the pathways of these three arms. Activation of the PERK pathway inhibits global protein synthesis via the phosphorylation of eukaryotic initiation factor 2 (eIf2α). ZIKV infected neuronal cells also show activation of the PERK pathway involving phosphorylation of eIf2α and activation of other targets such as ATF4, ATF3 and CHAC1*(*glutathione-specific γ-glutamylcyclotransferase 1) (204,205). Activation of IRE1α which has endoribonuclease activity splices out a 26-nucleotide intron from X-box binding protein 1 (XBP1) resulting in a frameshift. This spliced XBP1 is now a transcription factor and can translocate to the nucleus for the upregulation of UPR-related genes that are involved in protein folding and endoplasmic reticulum-associated degradation pathways (ERAD) (206). ZIKV is known to activate the IRE1α arm including *XBP1* gene splicing, ER degradation-enhancing α-mannosidase-like 1 (EDEM-1) activation in neuronal cells (204). Activation of IRE1α and *XBP1* gene-splicing facilitates lipid droplet production via stearoyl coenzyme A desaturase 1 (SCD1) (207). ER stress also induces the activation of ATF6 and it gets translocated from the ER to the Golgi apparatus where it is processed to expose its cytoplasmic DNA binding domain. This fragment of ATF6 processed in the Golgi apparatus acts by upregulating the expression of UPR related genes that aid in protein folding (208). ATF6 is known to positively regulate *XBP1* gene expression during UPR activation. Further, the ATF6 pathway is activated in ZIKV infected neuronal cells via its nuclear translocation (204).

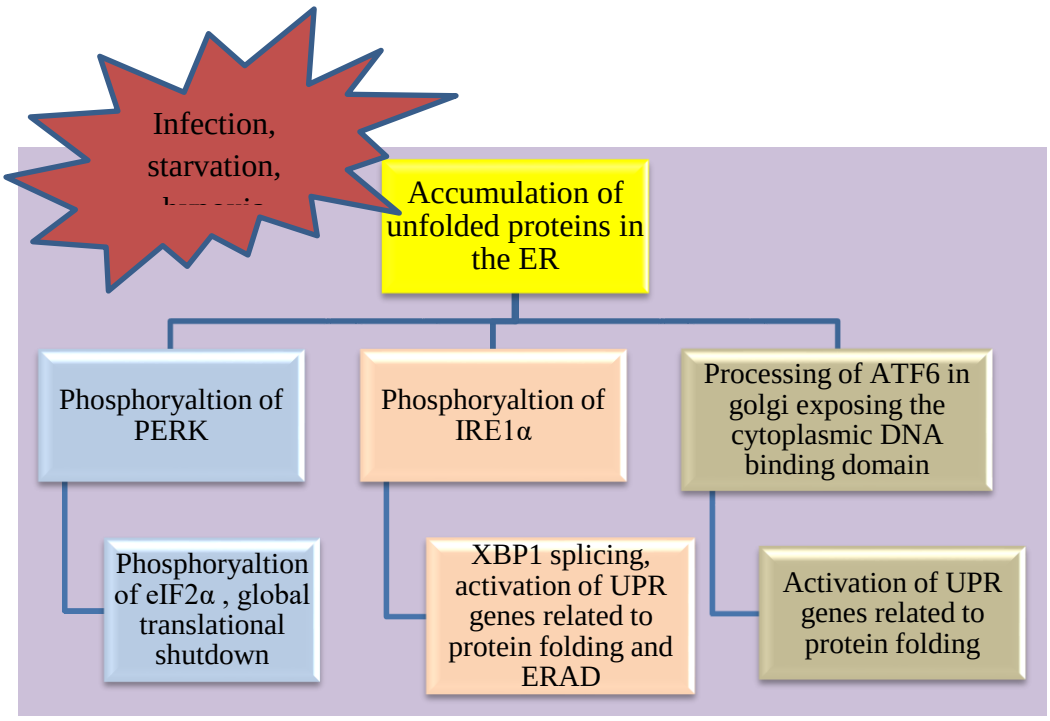

Fig.1.1. Schematic representation of the three arms of ER stress and its downstream targets.

Infection or starvation or hypoxia triggers unfolded protein accumulation in cells resulting in the activation and cross-talk among the three arms of ER stress leading to translation arrest, activation genes involved in protein folding and endoplasmic reticulum-associated degradation (ERAD).

Though cell survival is the main goal of signals generated through the three arms of ER stress, alternatively, persistent or sustained ER stress can activate apoptotic pathways as described in Figure 1.2. ER, stress-induced apoptosis is regulated by C/EBP homologous protein (CHOP), c-Jun N-terminal kinase (JNK) and B-cell lymphoma 2 (Bcl-2) family of proteins giving way to the activation of caspases (209). All three arms can cause transcriptional activation of CHOP. CHOP acts mainly by the downregulation of anti-apoptotic activity of Bcl-2 and also acts by upregulating other targets such as growth arrest and DNA damage-inducible gene 34 (GADD34), endoplasmic reticulum

oxidoreductin 1 alpha (ERO1α) and tribbles-related protein 3 (TRB3) (210-212). Activated JNK phosphorylates Bcl-2 and bim and results in the activation of bax and bak in the mitochondria, thereby initiating the intrinsic pathway of apoptosis (213). CHOP activation is also linked to ER stress and apoptosis in ZIKV infected neuronal cells (204). MAPK Kinases like JNK and p38 also play a critical role in the infection of flavivirus like dengue (214). Reticulophagy or ER-phagy cooperatively works with UPR to restore homeostasis in the ER membranes, but ZIKV can suppress this reticulophagy by reducing Family with Sequence Similarity 134 Member B (FAM134B) (215).

The cellular fate: undergoing ER stress

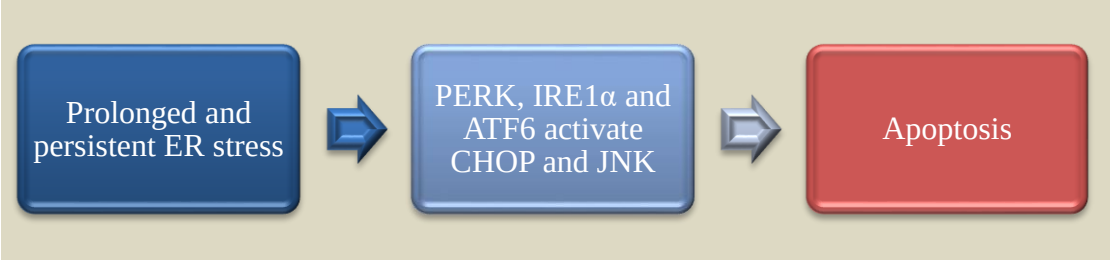

Fig.1.2. Schematic representation of pathways that leads the cell to progress from ER stress towards apoptosis.

ER stress in cells over some time results in activation of CHOP and JNK which drives the cells to undergo apoptosis by the activation of terminal caspases. Altogether, ZIKV can infect the placenta resulting in vertical transmission to the fetus and the underlying molecular mechanism involves prolonged ER stress leading to apoptosis.

1.16 Palmitoleate

The need for safe treatment or control methods in ZIKV infected pregnant women to prevent adverse effects in the developing fetus paved the pathway to study the use of palmitoleate, a nutrient compound as a potential therapeutic agent against ZIKV.

Structure and Sources: Palmitoleate is an omega 7 monounsaturated fatty acid (16:1n-7) with a double bond in the 7^{th} carbon atom counting from the methyl group with a total of 16 carbon atoms (216). It is found predominantly in adipose tissue and blood (217,218). Mammals can produce the cis form of palmitoleic acid synthesized from the saturated fatty acid palmitate via the stearoyl-CoA desaturase 1 enzyme (SCD1)(219). The trans-form of palmitoleic acid can be obtained from the consumption of dairy products and meat with fat content (219-221). Next to oleate, palmitoleate is the second most abundant monounsaturated fatty acid in the body (221). Plant sources of palmitoleate are macadamia nuts (*Macadamia integrifolia),* seabuckthorn (*Hippophae rhamnoides),* Durian fruits (*Durio graveolens) and blue-green algae* (221,222). Macadamia nuts alone contains 15-22% of palmitoleate occurring in its cis form (222-225).

Lipokine activity: Palmitoleate is a lipokine that can be released by fat tissue deposits, exerting functional roles in various organs (219). Palmitoleate released from fat tissue lysis during endurance exercise regimes is known to cause cardiomyocyte hypertrophy (226). Palmitoleate addition to bovine adipose tissue cultures promoted fatty acid oxidation and prevented lipogenesis (227). Similarly, systemic administration of palmitoleate reduced intramuscular fat deposition and improved insulin sensitivity in obese sheep (228). A macadamia nut-rich diet in humans has been shown to improve the lipid profile by decreasing low-density lipoprotein (LDL) (229,230). Supplementation of palmitoleate along with western diet in LDL receptor knock-out mice showed a significant reduction in the size of atherosclerotic plaques in the heart when compared to mice supplemented with high oleic acid olive oil or control mice fed with western diet alone (231). Further, palmitoleate can inhibit gap junction communication in vascular

endothelial cells (232). A cohort study involving people with different ethnic backgrounds in the USA found that high levels of circulating trans palmitoleate were correlated to a reduced risk of diabetes (220). Another cohort study in non-diabetic individuals also found a correlation between high levels of circulating palmitoleate and improved insulin sensitivity, as the palmitoleic acid can reduce fatty acid-induced damage which affects the beta cells of the pancreas and the glucose metabolism (233).

Positive effects on metabolic health: Palmitoleate could change the polarization of macrophages from the inflammatory M1 macrophage seen in animals fed with an obesogenic diet to the anti-inflammatory M2 macrophage through activation of adenosine monophosphate-activated protein kinase (AMPK) (234). The overall health benefits from palmitoleate supplementation in reducing inflammation, insulin resistance and preventing cardiovascular diseases could be attributed to the activation of AMPK supporting energy generation rather than its utilization (235). Palmitoleate supplementation reduces the inflammation in high-fat diet-induced fatty liver conditions (236,237). Palmitoleate was found to promote lipolysis via hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) activity in adipose tissue through peroxisome proliferator-activated receptor alpha (PPARα) activation (238,239). It was observed that the synthesis of palmitoleate was reduced in syncytiotrophoblasts isolated from obese mothers. This reduced palmitoleate production could aid in promoting an inflammatory environment and reduced insulin sensitivity in obese mothers (240). In a rodent, study palmitoleate was also found to augment the wound healing process, which could be due to its anti-inflammatory property (241). Palmitoleate was found to release satiety hormone cholecystokinin from the small intestine following oral supplementation in male rats

(242). Conversely, increased activity of the SCD1 enzyme that converts palmitic acid to palmitoleic acid results in insulin resistance, fatty liver and metabolic syndrome (243). Another study in male physicians observed that increased palmitoleate levels in the red blood cell (RBC) correlated to an increased risk of cardiovascular disease (244). When enveloped φ6 bacteriophages were treated with monounsaturated fatty acids such as palmitoleic acid and oleic acid, it resulted in inhibition of viral replication but saturated fatty acids like palmitic acid or myristic acid did not inhibit viral replication (245). Overall, palmitoleate has several arrays of functional roles in the body.

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1.18 Central hypothesis and specific aims

Central Hypothesis: It is known that ZIKV induces ER stress (Endoplasmic reticulum stress) in progenitor neuronal cells, a potential molecular mechanism behind microcephaly. Similarly, we also hypothesize that ZIKV- induced apoptosis in placental cells could be a downstream effect of sustained ER stress contributing to pathologies like IUGR and other obstetrical complications. Further, in our study, we try to address the protective role of palmitoleate against ZIKV-induced apoptosis in placental cells.

Specific Aim 1: To examine ZIKV induces ER stress and apoptosis in the placental trophoblast.

We hypothesize that ZIKV infection causes the activation of ER stress signaling pathway and activation of the apoptotic pathway in the placental trophoblast. We will be testing placental cells with ZIKV infection for activation of ER stress arms such as IRE1α and PERK and its downstream targets such as spliced forms of XBP1, phosphorylated eIF2α. We will also test the critical role of and activation ER stress in placental trophoblast apoptosis with ZIKV infection using small molecule inhibitors of ER stress like salubrinal. We will be testing other downstream targets that lead to apoptotic signals due to persistent ER stress such as CHOP and JNK activation.

Specific Aim 2: To elucidate the protective role of palmitoleate against ZIKV-induced apoptosis in placental trophoblasts.

We hypothesize that palmitoleate treatment protects against ZIKV-induced placental trophoblast apoptosis. We will test the protective role for palmitoleate supplementation against ZIKV-induced ER stress activation in an in vitro model using JEG-3, JAR and

HTR-8 cells. The protective role of palmitoleate could be lipid-based interaction with viral replication or by activation of cell survival factors.

CHAPTER 2: ZIKA VIRUS INFECTION INDUCES ENDOPLASMIC RETICULUM STRESS AND APOPTOSIS IN PLACENTAL TROPHOBLASTS

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2.1 Abstract

Zika virus (ZIKV) infection to a pregnant woman can be vertically transmitted to the fetus via the placenta leading to Congenital Zika syndrome. This is characterized by microcephaly, retinal defects and intrauterine growth retardation. ZIKV induces placental trophoblast apoptosis leading to severe abnormalities in the growth and development of the fetus. However, the molecular mechanism behind ZIKV-induced apoptosis in placental trophoblasts remains unclear. We hypothesize that ZIKV infection induces endoplasmic reticulum (ER) stress in the trophoblasts and sustained ER stress results in apoptosis. HTR-8(HTR-8/SVneo), a human normal immortalized trophoblast cell and human choriocarcinoma derived cell lines (JEG-3 and JAR) were infected with ZIKV. Biochemical and structural markers of apoptosis like caspase 3/7 activity and percent apoptotic nuclear morphological changes, respectively were assessed. ZIKV infection in placental trophoblasts showed an increase in the levels of CHOP mRNA and protein expression which is an inducer of apoptosis. Next, we also observed increased levels of ER stress markers such as phosphorylated forms of inositol-requiring transmembrane kinase/endoribonuclease 1α (P-IRE1α), and its downstream target, the spliced form of *XBP1* gene, phosphorylated eukaryotic initiation factor 2α (P-eIF2α), and activation of cJun N-terminal Kinase (JNK) and p38 mitogen activated protein kinase (MAPK) after

16-24h of ZIKV infection in trophoblasts. Inhibition of JNK or pan-caspases using small molecule inhibitors significantly prevented ZIKV-induced apoptosis in trophoblasts. Further, JNK inhibition also reduced *XBP1* gene splicing and viral E protein staining in ZIKV infected cells. In conclusion, the mechanism of ZIKV-induced placental trophoblast apoptosis involves the activation of ER stress and JNK activation and the inhibition of JNK dramatically prevents ZIKV-induced trophoblast apoptosis.

2.2 Introduction

Zika virus (ZIKV) infection reports were sporadic in the African continent but later gained significance due to 2015 endemic outbreaks. ZIKV was first isolated from a febrile macaque in the Ugandan Zika forest and only few cases of human infections were reported in early 1940s. ZIKV infection during pregnancy is associated with congenital Zika virus syndrome (CZS) (1). Infected infants manifest severe birth defects such as microcephaly, retinal defects and intrauterine growth retardation (IUGR) leading to still births as well as increased infant mortality after 2 to 3 days postpartum (2).

ZIKV is a single stranded positive sense RNA virus and its 10.8 kb genome can be directly translated into viral proteins. There are 3 structural proteins needed for assembly of viral particles such as envelope, precursor membrane protein and capsid protein, and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which aid in replication of the virus and are involved in attenuating host innate immune response (3-6). Virus size is roughly 50 nm with 180 copies of envelope and membrane proteins encompassing the outer structure. Envelope, the main structural protein covering

the virus forms raft configuration with three protein dimers arranged in parallel manner (7).

ZIKV infection is known to cause cell cycle arrest and apoptosis in progenitor neuronal cells, placental trophoblast and Hofbauer cells (8-11). Placental route plays a major role in disease transmission from the ZIKV infected mother to the fetus. The normal physiological role of the placenta, as a transient organ connecting the dam and the fetus is crucial for the fetal growth and survival through several functions like nutrient transport, respiratory gas exchange and metabolism of waste products (12). Stromal cores in the chorionic villi includes the fetal blood vessels and Hofbauer cells (placental resident macrophages) which regulate the branching of the villous (13). Recent studies suggest that placental epithelial cells and the other placental cells such as endothelial cells and Hofbauer cells can also be infected and results in dissemination of ZIKV from mother to fetus (14-16).. ZIKV was found to actively replicate in placental fibroblasts and Hofbauer cells resulting in spread of the infection to the neuronal progenitor cells of the fetal brain (17). Further, T cell immunoglobulin and mucin domain 1 (TIM1) (18)which are considered as important cofactor for the entry of ZIKV into cells is highly expressed in cytotrophoblasts, invasive cytotrophoblasts, placental fibroblasts, umbilical vein endothelial cells, hofbauer cells and amniochorionic membranes(16,19). Other cofactors such as Tyro3 and Axl (18,20), implicated in ZIKV entry are also variably expressed in the placental cells (16). In the present study, we show evidence for ZIKV infection to placental trophoblast results in ER stress and apoptosis via MAPK activation.

2.3 Materials and methods

Cells: HTR-8/SVneo (HTR-8), normal human immortalized first-trimester placental trophoblast cells, and choriocarcinoma-derived third-trimester placental trophoblast cell lines (JEG-3 and JAR) were used. JAR and HTR-8 cells were cultured in DMEM containing 10% fetal bovine serum, 0.01% plasmocin. JEG-3 cells were cultured in MEM containing 10% fetal bovine serum, and plasmocin (0.01%). All the cells used the present study were obtained from ATCC and periodically tested for mycoplasma

Antibodies: Primary antibodies against ZIKV E protein (# GTX133314) from Gene Tex, Inc. CA, eIF2α (# 5324), p-eIF2α (# 3388), p-p38 (# 9211),p38 (# 9212), p-JNK(# 9251), JNK (# 9252), IRE1α(# 3294), CHOP (# 2895), Bip (# 3183), HDAC1(# 34589), HERPUD1(# 26730), GADD45 (# 46325) and BCl2 (# 15071) were purchased from Cell Signaling Technologies, MA. Phospho-IRE1α antibody (# ab48187) was obtained from abcam and Actin antibody (# A-5441) was from MilliporeSigma, MA. Rabbit HRP-conjugated secondary antibody (#111-035-144) and mouse HRP-conjugated secondary antibodies (# 715-035-150) were obtained from Jackson ImmunoResearch, PA. Alexa flour 488 conjugated anti-rabbit antibody (# A11008) and Alexa flour 594 conjugated anti-mouse antibody (# A-11032) were obtained from Invitrogen,CA.

Viral Strains and trophoblast infection: MR766 strain (originally isolated from sentinel monkey in Uganda), recombinant MR strain (r-MRV) of Zika virus is generated as described (21), and PRVABC-59 strain (Asian lineage derived American strain from 2015 outbreak in Puerto Rico) were used in the present study. Trophoblasts were infected with 0.1 -1 multiplicity of infection (MOI) in the infection media MEM (Gibco) containing 2% fetal bovine serum, streptomycin, penicillin, 7.5% sodium bicarbonate,

20mM HEPES, 1 mM sodium pyruvate, and 1x nonessential amino acids and 0.01% plasmocin for 1 to 2h. After virus adsorption, the media was replaced with DMEM or MEM containing 10% fetal bovine serum and 1% BSA.

Characterization of apoptosis: Biochemical and structural markers of apoptosis like caspase 3/7 activity and percent apoptotic nuclear morphological changes, respectively. Caspase 3/7 (effector caspase) activity was measured by enzymatic fluorophore release (Apo-One) according to the manufacturer's instructions (Promega, Madison, WI #G7791) and reported as fold change compared to vehicle treatment or mock infection, with experiments performed in quadruplicate. Cells were seeded in 24 well plates, infected with ZIKV for 48 to 72h and percent apoptosis was quantified by characteristic nuclear morphology as described (22) and visualized by treatment with the fluorescent DNA-binding dye, DAPI (4', 6-diamidine-2-phenylindole dihydrochloride) as described (22). Briefly, cells were stained with 5 μg/mL of fluorescent DNA-binding dye, 4', 6-diamidine-2-phenylindole dihydrochloride (DAPI) for 20 minutes at 37ºC. Apoptotic nuclei (condensed, fragmented) were counted and presented as a percent of total nuclei. At least 100 cells were counted per well and experiments were performed in triplicate.

Quantitative real time Polymerase Chain Reaction: Cells were infected with ZIKV and the total RNA was isolated using TriZOL reagent (Thermo Scientific). Around 1-5 μg RNA from each sample was reverse transcribed to cDNA with random hexamers, RNAse OUT and Superscript II reverse transcriptase with low RNAse H activity (Invitrogen). CHOP mRNA and 18S rRNA were quantified using primers listed in table 2.1 as described (23).

Table 2.1 Primer details

Primer	Forward primer	Reverse primer	Product length
XBP1	5'AAACAGAGTAGCAG C TCAGACTGC 3'	5'TCCTTCTGGGTAGAC CTCTGGGAG 3'	unspliced forms`~474bp cleaved by the restriction endonuclease two products are around 296bp and 183bp spliced forms lack restriction enzyme site ~ 448bp
GAPDH	5'AATCCCATCACCATC TTCCA 3'	5'TTCACACCCATGACG AAC AT 3'	~194bp
18srRNA	5'CGTTCTTAGTTGGTG GAGCG 3'	5'CGCTGAGCCAGT CAG TGTAG 3'	~212bp

***XBP1* splicing assay:** Approximately 5µg total RNA diluted 1:3 (JEG-3 and JAR cells) or 1:10 (HTR-8) was used for cDNA synthesis. Then it was subjected to PCR to amplify *XBP1* gene using the primer set (each 20µM) as described (24). Around 8 µL of the obtained PCR product was digested with 20 U of PstI (New England Bio labs) in 1µLNEB buffer containing 100 mM NaCl, 50 mM Tris-HCl,10 mM $MgCl_2$ and 100 µg/mL BSA and incubation was done at 37^{o}C for 2h. The restriction enzyme digested PCR product was electrophoresed in 2% agarose gel stained with ethidium bromide. The unspliced forms are around 474 bp and the restriction endonuclease digested products were 296bp and 183bp. The spliced forms lack restriction enzyme site so the bands are

visualized around 448bp. GAPDH was used as control and was amplified by the primers listed in table 2.1.

Western blot analysis: Cell lysates were prepared by adding 100µL of lysis buffer containing 50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM DTT, 1mM Na_3Vo_4, 1mM PMSF, 100mM NaF and 1% Triton x-100. Cells were scraped, incubated for 40 minutes in ice and cell supernatant was prepared after 10,000 x g for 10 min of centrifugation. Protein estimation was done using modified Lowry method using Pierce 660nm protein assay reagent (ThermoFisher Scientific). Around 30µg protein was separated in a 10% polyacrylamide gel or 4 to 20% gradient gel and then transferred into nitrocellulose membrane. The membrane was blocked with either 5% skim milk powder in TBST or with 5% BSA in TBST. Primary antibody was used in 1: 1000 dilution in 5% skim milk powder in TBST or with 5% BSA in TBST. Secondary antibody was used in 1:500 dilution. Washes of 10 mins for 3 times were given after both primary and secondary antibody incubation. The blots were developed using Clarity Western ECL substrate or Clarity Max ECL substrate (Bio-Rad).

Isolation of nuclear proteins: Cells were scraped in buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1 mM DTT, 0.5% nonidet-P40 substitute (Sigma) and protease inhibitor and cell lysate were incubated for 10min in ice. Cell lysates were centrifuged at 15,000 x g for 3 mins. The supernatant has cytoplasmic proteins and to the settled precipitated nuclear contents 50-100 µL of buffer B containing 20mM HEPES, 0.4M NaCl, 1mM EDTA, 0.05mM DTT, 10% glycerol and proteinase inhibitor was added and incubated in ice with intermittent vortexing for 40 mins and then centrifuged at 15,000g for 5 mins to collect the nuclear protein lysate in the supernatant.

Immunofluorescence analysis: Placental trophoblast was infected with ZIKV, 0.1 MOI for 48-72h. Infected cell media was aspirated and fixed with methanol and acetone (ratio of 1:1) and washed with PBS. Blocking was done using solution containing 1% BSA and 22.52 mg/mL glycine in PBST. Zika virus envelope protein (E protein) rabbit polyclonal was used at a dilution of 1:500 or CHOP mouse monoclonal antibody at a dilution of 1:400 was used and incubated overnight at 4^{o}C. After primary antibody incubation, cells were washed thrice with PBS with an interval of 5 mins each. Alexa flour 488/594 conjugated secondary antibody was added at a dilution of 1:1000 and kept in a shaker at room temperature for 2h. After incubation, the cells were washed thrice in PBS, counter stained with DAPI (0.3 µM) for 10mins at 37^{o}C and washed with PBS. Images were taken using EVOS FL microscope (Thermo Fisher) or Nikon A1R-Ti2 confocal system**.**

Statistical analysis: Analysis of variance (ANOVA) with post-hoc Bonferroni corrections was employed for comparisons of multiple groups and Student's t-test were performed for comparisons between two groups. P value <0.05 was considered statistically significant.

2.4 Results

Zika Virus infection induces placental trophoblast apoptosis: We performed immunofluorescence analysis in placental trophoblasts (HTR-8 cells) infected with 0.1 MOI of r-MRV or PRV strain of ZIKV. We observed viral E protein staining after 72h in ZIKV infected trophoblasts, while there was no staining observed in vehicle or mock infected trophoblast cells (Fig. 2.1A). We also analyzed the expression of viral E protein after 8-24h of infection in JEG-3, JAR and HTR-8 cells. Viral E protein levels were

dramatically increased at 16 or 24h post infection of ZIKV in JEG-3, JAR and HTR-8 cells compared to mock infected cells. The levels of beta-actin were used as control and remains unchanged at different timepoints of infected cells and mock infected trophoblasts (Fig 2.1B).

Next, we assessed biochemical characteristics of apoptosis such as caspase 3 and 7 activation and nuclear morphological changes with ZIKV infection in placental trophoblast cells. Both first trimester-derived trophoblast (HTR-8) and term-derived trophoblast cells (JEG-3) cells infected with 1.0 MOI of MRV (MR766) and PRV strains of ZIKV, 48h post infection showed a significant increase caspase 3 and 7 activity and percent apoptotic nuclei indicating apoptosis (Fig. 2.2A). We also assessed trophoblast apoptosis with 0.1 MOI of r-MRV strain after 48-72 hours of infection and, we observed a significant increase in caspase 3/7 activity and percent apoptotic nuclei in placental trophoblasts (JEG-3, JAR and HTR-8) (Fig. 2.2B-D). We also observed a trend in downregulation of anti-apoptotic Bcl_2 protein with 0.1 MOI of r-MRV strain of ZIKV 16-24 h post infection in JEG-3 and HTR-8 cells (Fig S1). These data suggest that ZIKV infection of placental trophoblast cells induces apoptosis.

Figure 1A

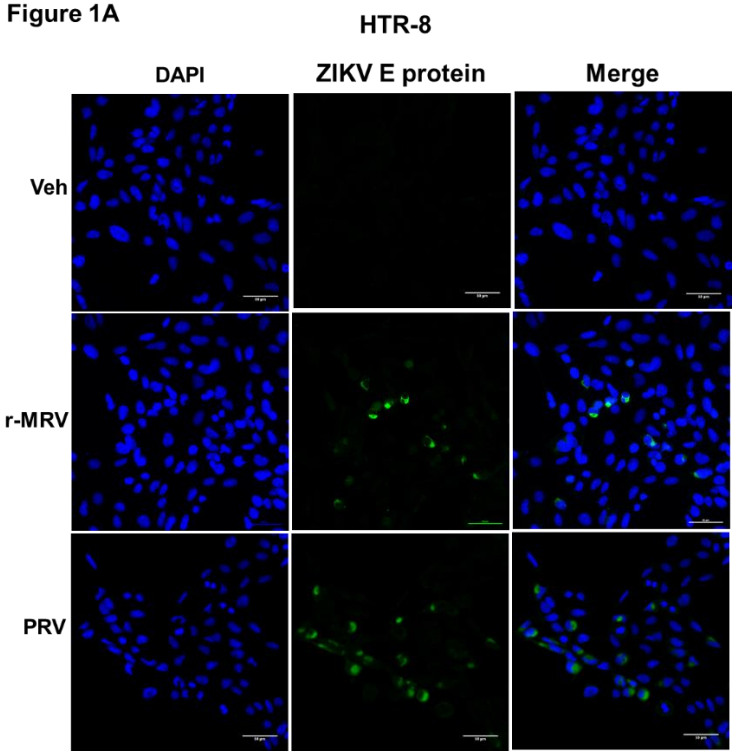


Figure 1B

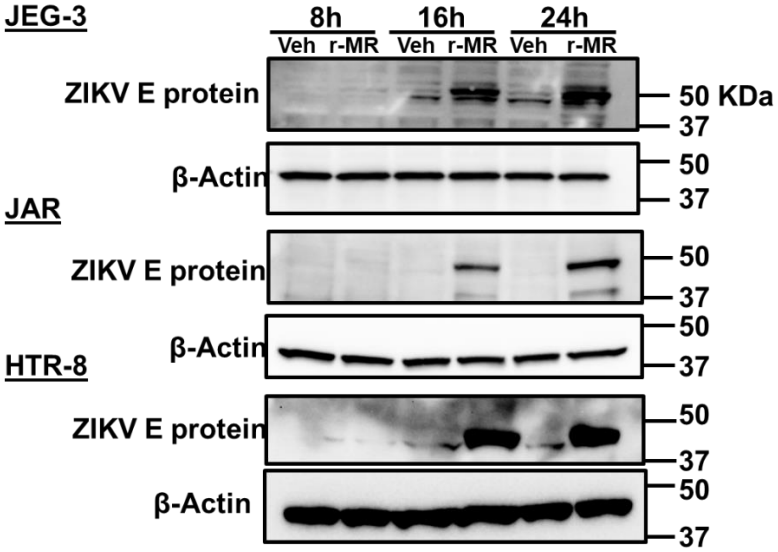
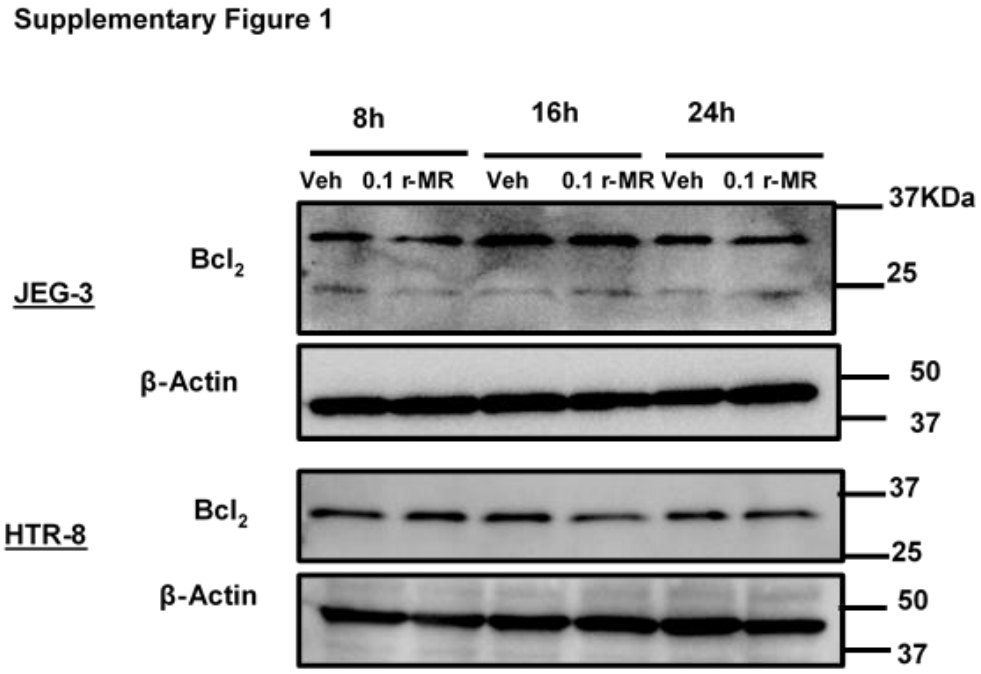


Fig.2.1. ZIKV infects placental trophoblasts.

(A) Immunofluorescence staining of Viral E protein in HTR-8 (HTR-8/SVneo) cells 72h post infection with 0.1 multiplicity of infection (MOI) recombinant MR strain (r-MRV) or PRVABC-59 (PRV) or uninfected vehicle cells. Nuclei were stained with DAPI and ZIKV E protein stained with rabbit polyclonal primary antibody and Alexaflour 488-conjugated ant-rabbit secondary antibody. (B) Western blot analysis showing increased expression of viral E protein, 16-24h post infection when compared to uninfected vehicle cells in JEG-3, JAR and HTR-8. β-Actin was used as a loading control.



Supplementary Fig.1. ZIKV infection results in downregulation of anti-apoptotic Bcl2. Representative western blot showing anti-apoptotic Bcl2 is downregulated in JEG3 and HTR-8 cells, 24h and 16h post-infection respectively.

Figure2

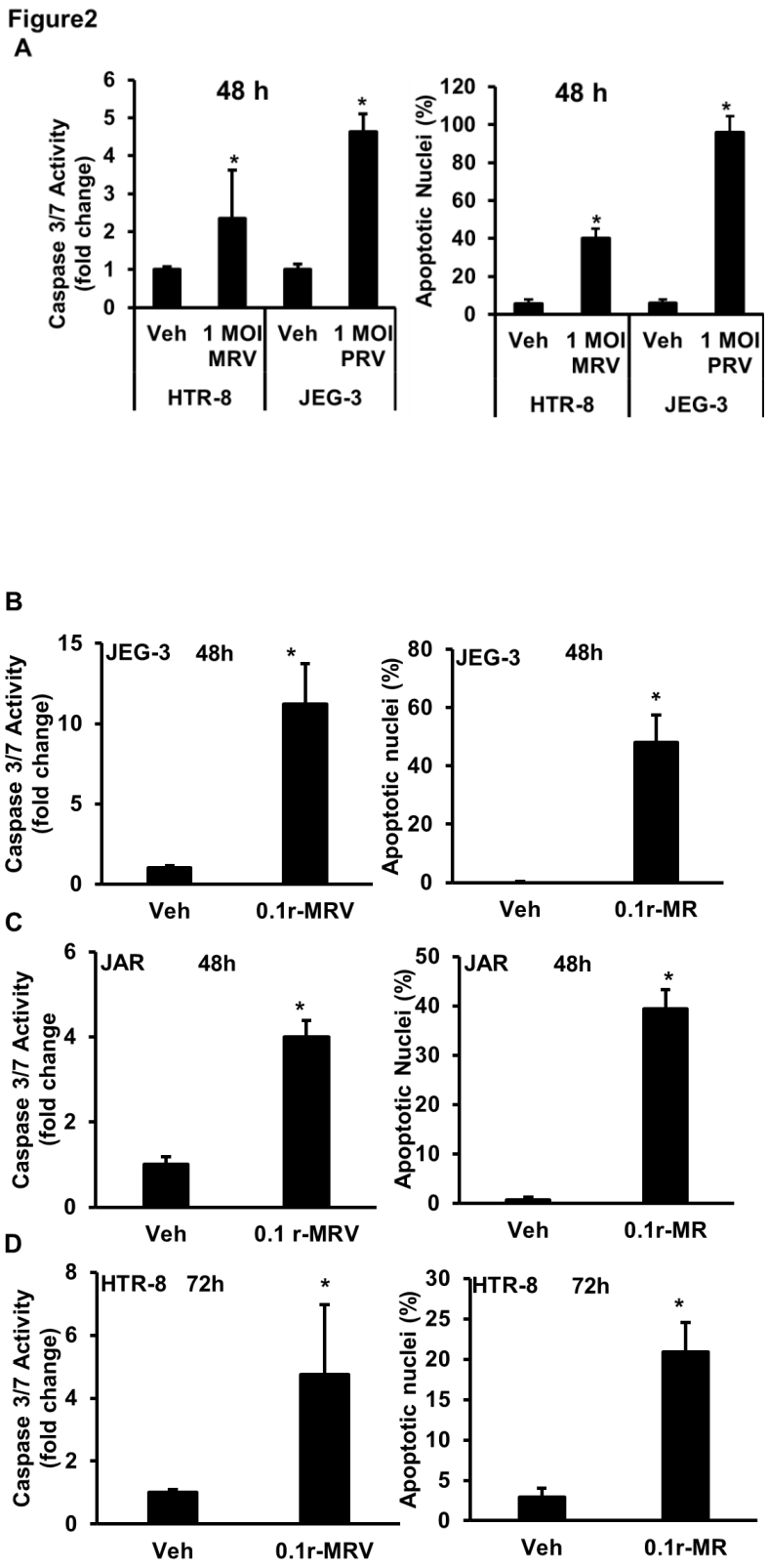

A
48 h
Caspase 3/7 Activity (fold change)
Apoptotic Nuclei (%)
Veh
1 MOI MRV
1 MOI PRV
HTR-8
JEG-3
B
JEG-3 48h
Caspase 3/7 Activity (fold change)
Apoptotic nuclei (%)
Veh
0.1r-MRV
0.1r-MR
C
JAR 48h
Caspase 3/7 Activity (fold change
Apoptotic Nuclei (%)
0.1 r-MRV
D
HTR-8 72h

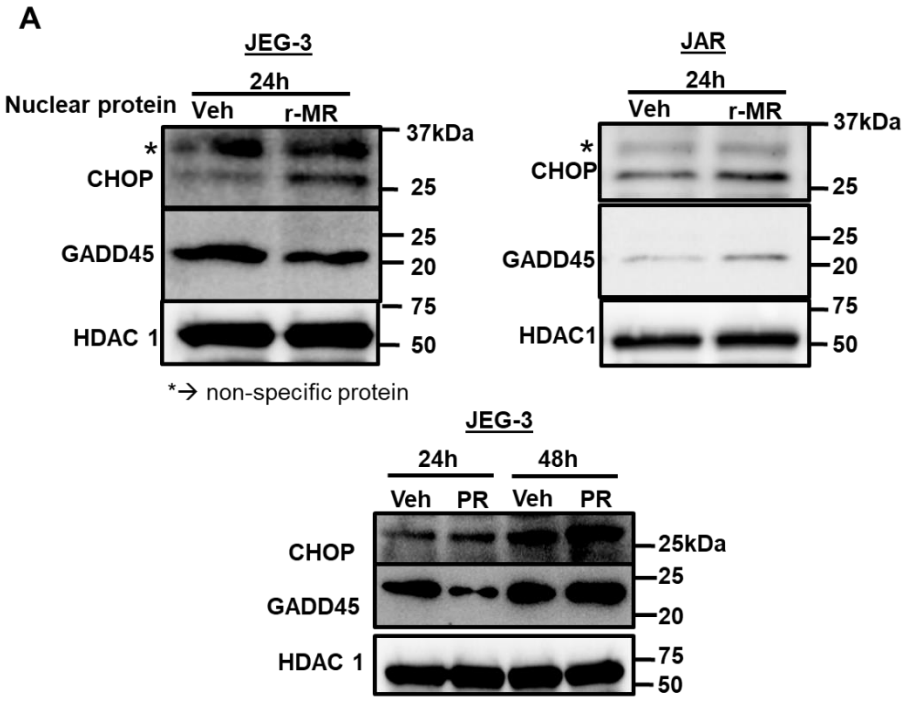
Fig. 2.2. ZIKV infection induces placental trophoblast apoptosis.

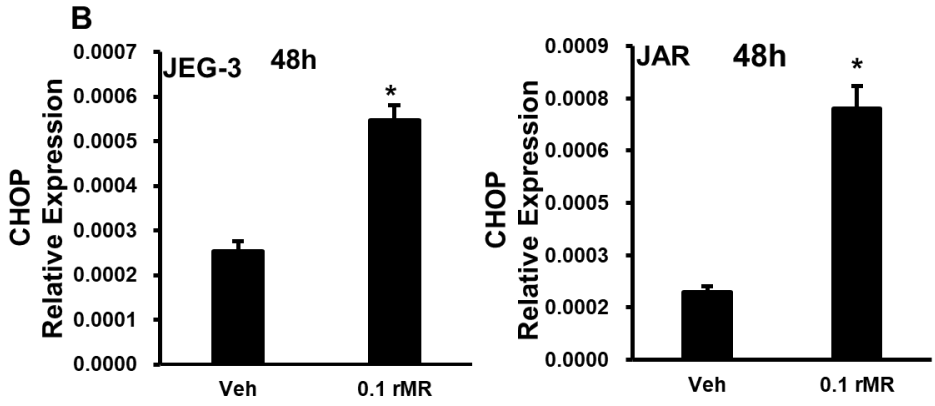
(A) JEG-3 and HTR-8 infected with 1.0 MOI MRV or PRV showing significant increase in caspase 3/7 activity when compared to uninfected vehicle cells (left panel). Similarly, ZIKV infection with 1.0 MOI MRV or PRV also showed increased percentage apoptotic nuclei compared to uninfected vehicle cells (right panel). Similarly, ZIKV infection with 0.1 MOI r-MRV to placental trophoblast also induces apoptosis as evidenced by an increase in caspase 3/7 activity and percent apoptotic nuclei and in JEG-3 (B), JAR (C) and HTR-8 (D) cells compared to uninfected vehicle cells. Each value presents mean ± SEM of biological replicates (n=4) for caspase 3/7 activity and (n=3) for percentage apoptotic nuclear morphology, *P<0.05 compared to uninfected vehicle cells; statistical comparison by Student's t-test.

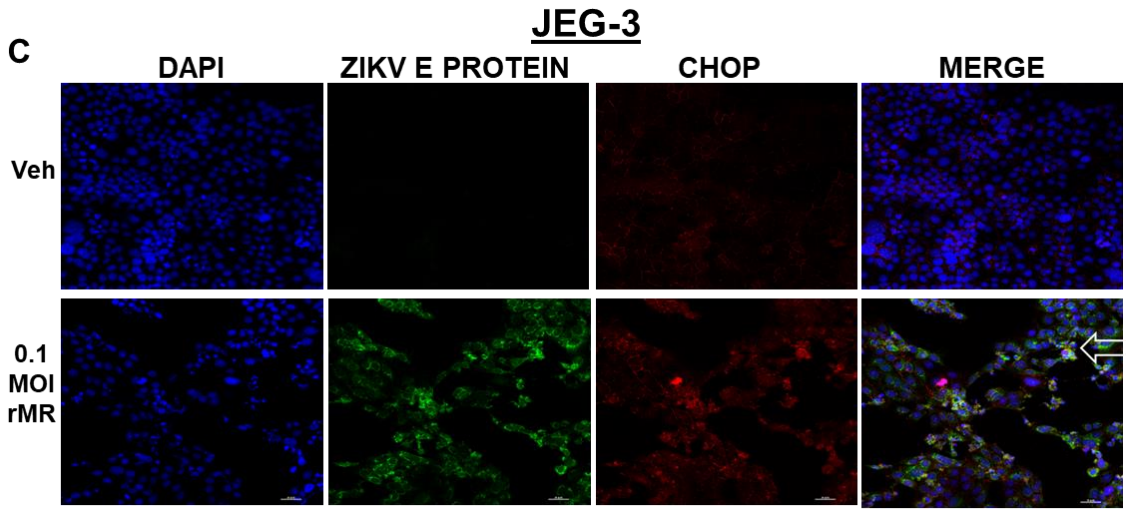
ZIKV infection in trophoblasts induces CHOP nuclear translocation, a transcription factor that can activate apoptosis: Sustained ER stress can progress the cells into apoptosis either via CHOP or JNK activation (25). Active and spliced *XBP1* can trancriptionally increase expression of ER stress response proteins like CHOP (26). We examined whether ZIKV infection can induce the expression of other downstream targets that activates apoptosis. We tested the nuclear translocation of CHOP with 0.1 MOI r-MRV ZIKV infection in trophoblasts and confirmed that there was increased nuclear translocation of CHOP protein after 24h ZIKV infection in JEG and JAR cells (Fig. 2.4A). Similarly, there was a trend in increase in the levels of CHOP mRNA, 24h post infection in both JAR and JEG-3 cells where as it was 8h post infection in HTR-8 cells (Fig. S2). We also observed a significant increase in CHOP mRNA expression 48h post infection compared to uninfected vehicle cells in both JEG-3 and JAR cells (Fig. 2.4B). We further confirmed CHOP nuclear translocation in JEG-3 (Fig. 2.4C) and JAR cells (Fig 2.4D) using immunofluorescence analysis. We observed enhanced CHOP nuclear localization surrounded by viral E protein staining in the cytoplasm of r-MRV infected cells at 48h post infection. We also examined whether ZIKV infection induces

Growth Arrest and DNA Damage-inducible 45 (GADD45) which can activate and promote apoptosis. Interestingly, we found that the nuclear levels of GADD45 were increased after 24h of ZIKV infection in JAR cells and JEG-3 cells (Fig. 2.3A). Further, JEG-3 cells infected with PRV 24-48 h post infection showed increased nuclear translocation of CHOP (Fig 2.3A, bottom panel). However nuclear levels of GADD45 were increased only after 48 h of infection with PRV, when compared to uninfected vehicle cells (Fig 2.3A, bottom panel). These data suggests that both MRV and PRV similarly induced CHOP and GADD45 nuclear translocation.

Figure 3




B
JEG-3 48h
CHOP Relative Expression
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
*
Veh
0.1 rMR
JAR 48h
CHOP Relative Expression
0.0009
0.0008
0.0006
0.0005
0.0003
0.0002
0.0000
*
Veh
0.1 rMR


JEG-3
C
DAPI
ZIKV E PROTEIN
CHOP
MERGE
Veh
0.1 MOI rMR

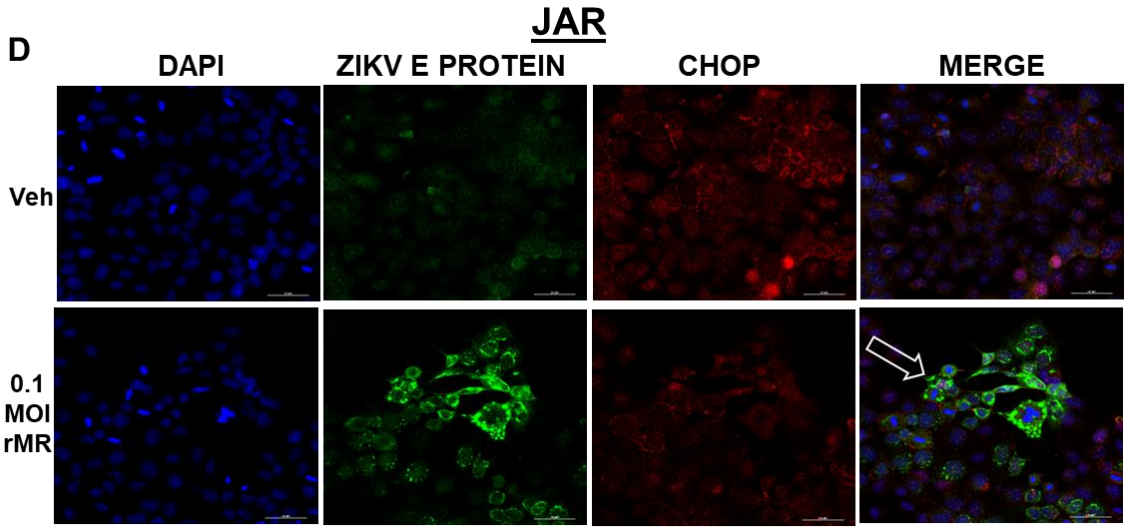
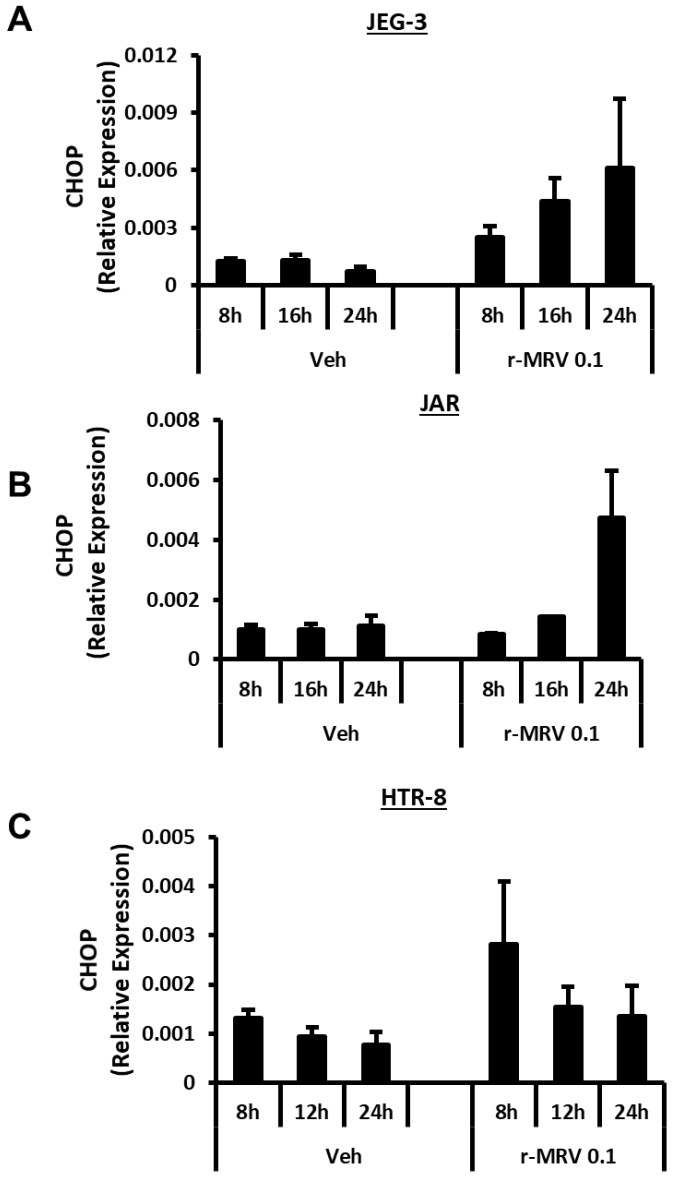

JAR
D
DAPI
ZIKV E PROTEIN
CHOP
MERGE
Veh
0.1 MOI rMR

Fig. 2.3. ZIKV infection induces the expression CHOP, a transcription factor that activate apoptosis.

(A) Immunoblot analysis showed an increase in the expression of CHOP in nuclear extracts of ZIKV infected cells (0.1 MOI r-MRV) compared to vehicle uninfected cells in JEG-3 (left panel) and JAR cells (right panel), increased nuclear levels of CHOP can act as an initiator for apoptosis. JEG-3 (left panel) and JAR cells (right panel). We also observed increased GADD45 levels in nuclear protein, which is produced in response to DNA damage, after 24h of infection in JAR cells (right panel) with 0.1 MOI of r-MRV compared to uninfected vehicle cells and this increase of GADD45 in the infected cells was absent in JEG-3 (left panel). HDAC1 was used as a loading control. We also observed increase in expression of CHOP nuclear protein both 24 and 48h post infection and GADD45 nuclear protein around 48h post infection in JEG-3 cells with 0.1 MOI PRV (lower middle panel). (B) JEG-3 (left panel) and JAR (right panel) cells after 48h of 0.1 MOI of r-MRV showed significant increase in CHOP mRNA levels compared to uninfected vehicle cells. CHOP mRNA expression were reported relative to 18S rRNA. Each value presents mean ± SEM of biological replicates (n=3), *P< 0.05 compared to uninfected vehicle cells; statistical comparison by Student's t-test. (C) Immunofluorescence analysis was also performed to test CHOP nuclear translocation by using Viral E protein staining indicated as green signal and CHOP as red signal. (C) JEG-3 cells: uninfected vehicle cells showed no viral E protein staining but has endogenous CHOP expression. Cells infected with 0.1MOI r-MRV show nuclear CHOP expression surrounded by viral E protein staining in the cytoplasm in the merged panel. (D) JAR cells: uninfected vehicle cells show no viral E protein staining but endogenous CHOP expression is seen. Cells infected with 0.1MOI r-MRV showed increased nuclear CHOP expression surrounded by viral E protein staining in the cytoplasm showed in the merged panel indicated in white arrow. Scale bar represents 50 μM size.

Supplementary Figure 2

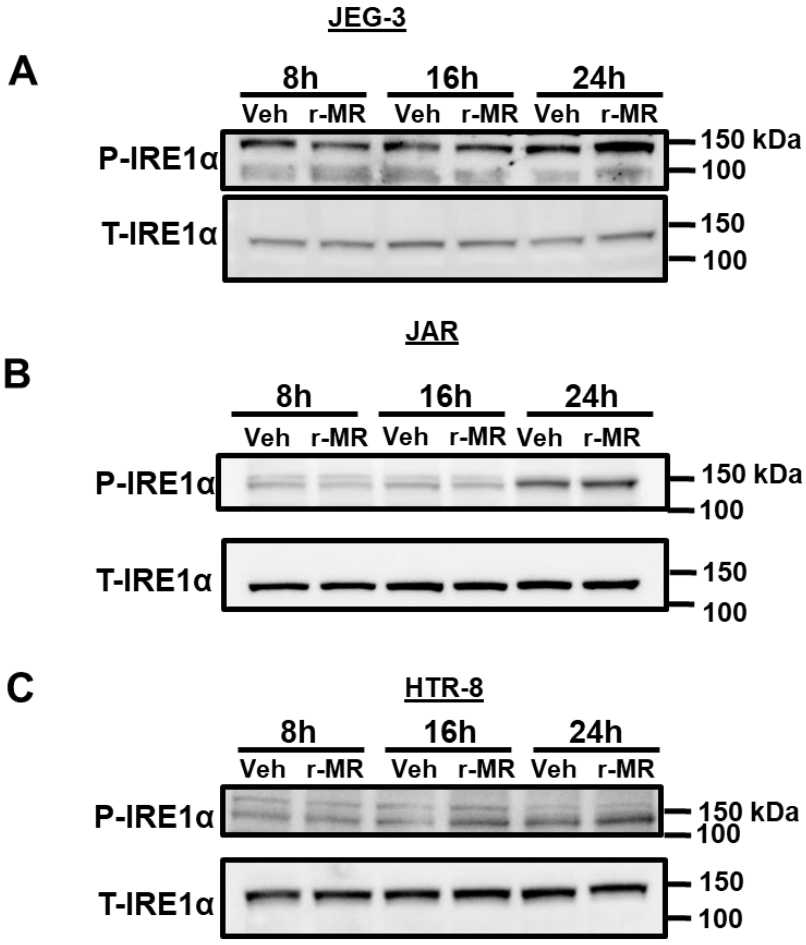


Supplementary Fig.2. CHOP expression in ZIKV infected placental trophoblasts. CHOP mRNA expression is showing a trend towards increase around 24h post infection with 0.1 r-ZIKV MR strain in JEG-3 (A), (B) JAR and (C) HTR-8 compared to uninfected or mock-infected vehicle cells.

ZIKV infection induces ER stress in placental trophoblasts: We examined the activation of IRE1α arm of ER stress with ZIKV infection in placental trophoblasts. We observed an increase in the levels of phosphorylated IRE1α in placental trophoblasts after

24h of ZIKV infection, suggesting that the ER stress sensor proteins are activated in JEG-3, JAR and HTR-8 (Fig 2.4 A-C). However, the levels of total IRE1α protein were unchanged (Fig. 2.4 A-C). Activated IRE1α, an endoribonuclease is known to enhance the splicing and processing of *XBP1* mRNA. We next examined the downstream target of activated IRE1α that is enhanced splicing of *XBP1* gene. We observed a dramatic increase in the levels of *XBP1* gene splicing 8- 24h post infection in JEG-3, JAR and HTR-8 cells (Fig. 2.4D-E). JEG-3 and JAR cells showed a time dependent increase in the active spliced form of *XBP1* which was dramatically increased in 24h. In HTR-8 cells, there was active form of spliced *XBP1* particularly evident at 8h post infection. GAPDH was used as loading control. ER stress has been shown to block global protein translation (27). Host immune defense such as antiviral response by interferons are also known to activate eIF2α via phosphorylation (28). We assessed the activation of eIF2α via phosphorylation. ZIKV infection of placental trophoblast showed increased levels of phosphorylated eIF2α (P-eIF2α) after 24 hours of infection in JEG-3 and HTR-8 cells whereas activation of eIF2α was observed after 16h of infection in JAR cells and the levels of total eIF2α remain unaltered (Fig.2.5A). Increased P-eIF2α levels can block global protein translation in the placental trophoblasts with ZIKV infection. We also found that Bip/GRP78, regulator of ER stress is slightly increased 24h post infection in JEG cells but this increase did not correspond to JAR and HTR-8 cells with ZIKV infection (Fig.2.5B). Homocysteine Inducible ER Protein with ubiquitin like domain 1 (HERPUD1) expression also remained unchanged in both vehicle and r-MRV infected JEG-3, JAR and HTR-8 cells (Fig.2.5C).

Figure 4


JEG-3
A
8h
16h
24h
Veh
r-MR
Veh
r-MR
Veh
r-MR
P-IRE1α
150 kDa
100
T-IRE1α
150
100
JAR
B
8h
16h
24h
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r-MR
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P-IRE1α
150 kDa
100
T-IRE1α
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100

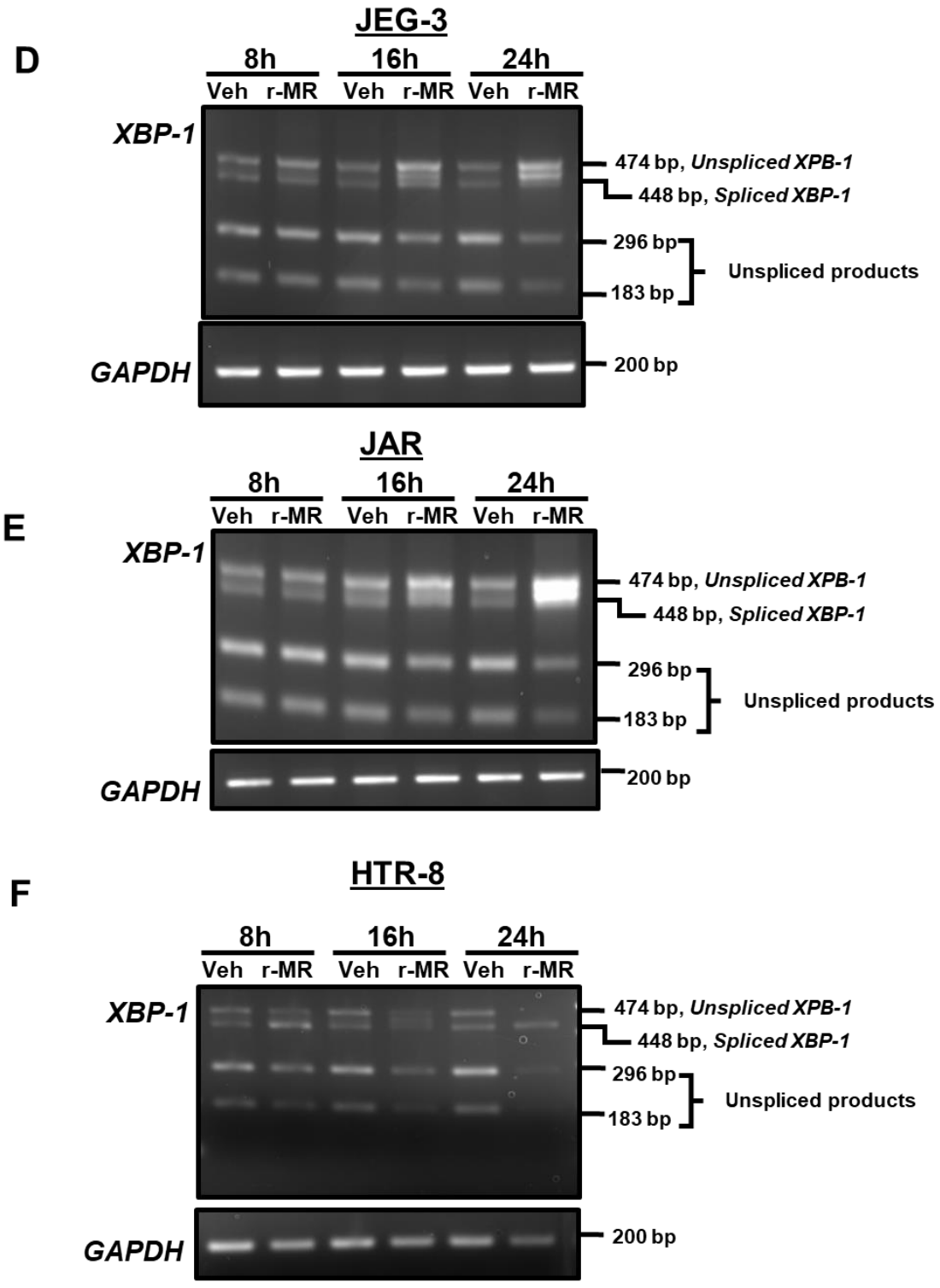


Fig.2.4. ZIKV infection activates IRE1α arm of ER stress in placental trophoblasts.

(A) Western blot images shows increased levels of phosphorylated IRE1α after 24 hrs of infection with 0.1 MOI r-MRV in JEG-3 cells. (B) A slight increase in phospho-IRE1α

was observed in JAR cells after 24h of infection and HTR-8 cells also showed increased phospho- IRE1α after 16-24h of ZIKV infection compared to uninfected vehicle cells. Trophoblasts were infected with 0.1 MOI r-MR and XBP1 splicing was observed after 8, 16 and 24 hrs of infection. (C) JEG 3 cells showed an increased spliced form of XBP1 mRNA at 24h post infection. (D) JAR cells infected with 0.1 MOI r-MR strain showed increased levels of spliced XBP1 at 24h post infection compared to mock-infected or uninfected vehicle cells. (E) HTR-8 cells infected with 0.1 MOI, r-MR showed increased XBP1 spliced form starting from 8 h post infection and stayed until 24 hr post infection. The unspliced XBP1 cDNA is cleaved by PstI restriction enzyme and shows faster migration pattern than the spliced form. GAPDH was amplified as a loading control.

Figure 5

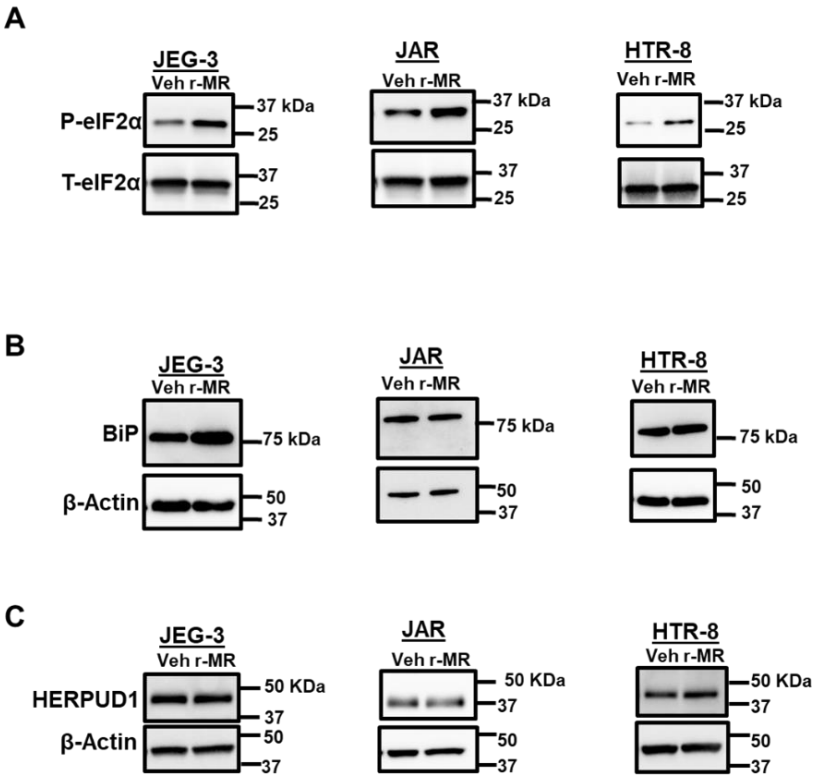
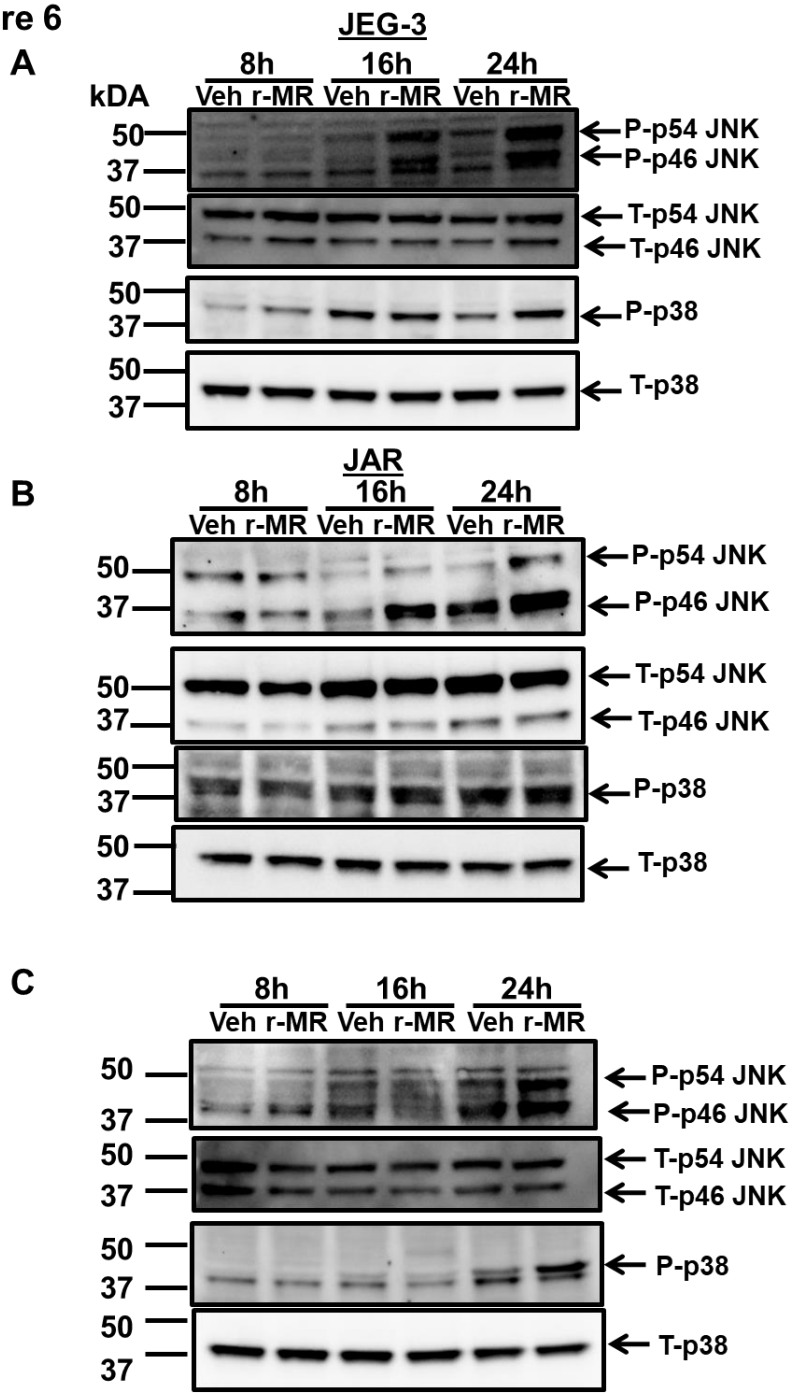


Fig.2.5. ZIKV infection and ER stress markers in trophoblasts.

(A) Western blot showing increase in the expression of phosphorylated eIF2α after 24h of infection with 0.1 MOI r-MRV in both JEG-3 and HTR-8 (HTR-8 SV/neo) cells and 16h post infection in JAR cells compared to uninfected vehicle cells. Total eIF2α levels remained constant. (B) ZIKV infected trophoblast also showed an increase in the levels of Bip/GRP78 in JEG-3 but not in JAR and HTR-8 cells compared to uninfected vehicle cells. (C) HERPUD1 levels were unchanged in uninfected and ZIKV infected JEG-3, JAR and HTR-8 cells. Actin was used as a loading control.

Activation of Mitogen Activated Protein Kinase (MAPK): ER stress-induced activation of IRE1α has been shown to activate JNK (29,30). To assess the mechanism of ZIKV-induced placental trophoblasts, we sought to test the activation of JNK, which can initiate apoptotic cell death during sustained ER stress. ZIKV infection in JEG-3 cells showed increased levels of JNK phosphorylation after 16-24 h of infection suggesting an activation of JNK (Fig.2.6A). Similarly, we also observed an increase in phospho-JNK levels in JAR and HTR-8 cells, 24 h post infection in comparison to the total JNK levels (Fig.2.6 B, C). We also tested 0.1 MOI of PR strain infection in JEG-3 cells and observed increased phosphorylation of JNK, 24h and 48h post infection (Fig.2.6D) when compared to uninfected vehicle cells. These data provide insights on the mechanism of ER stress and its contribution to the apoptotic signal. p38 MAPK is known have a regulatory control on CHOP (31), also p38 activation in DENV is known to be associated with hepatic damage(32). Here we found that ZIKV infection also activates p38 MAPK via phosphorylation in JEG-3, HTR-8 cells after 24h of infection (Fig.2.6A, C) and in JAR cells it was more prominently after 16h of infection (Fig.2.6B). These data suggest that ZIKV infection induces p38 MAPK activation.

Figure 6


A
JEG-3
8h
16h
24h
kDA
Veh r-MR Veh r-MR Veh r-MR
50
37
P-p54 JNK
P-p46 JNK
T-p54 JNK
T-p46 JNK
P-p38
T-p38
B
JAR
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24h
Veh r-MR Veh r-MR Veh r-MR

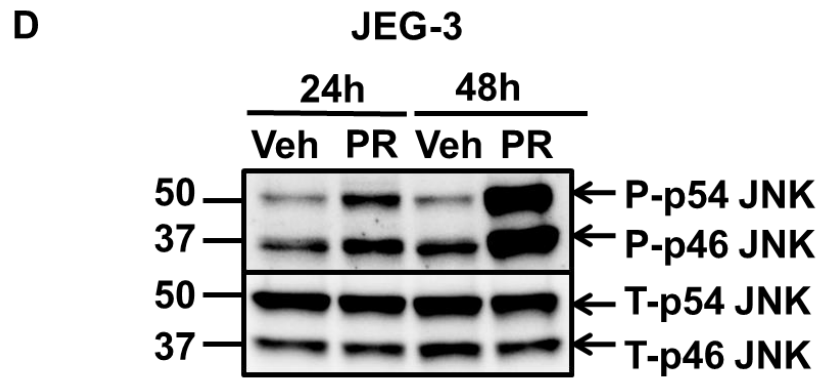


Fig.2.6. Activation of Mitogen Activated Protein Kinase (MAPK) in ZIKV infected trophoblasts.

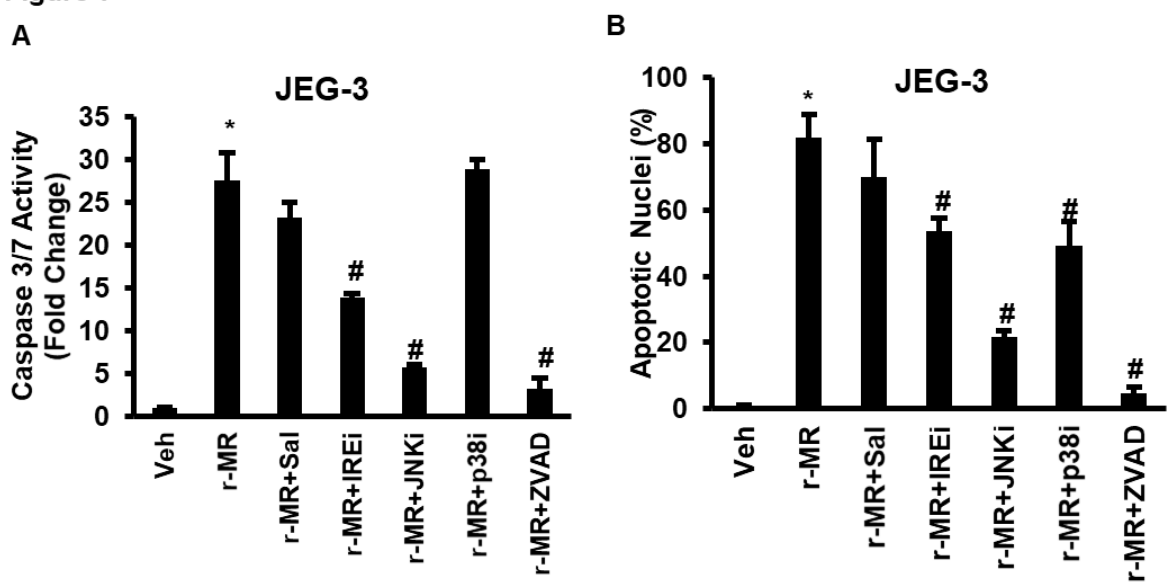
(A) Immunoblot analysis of JNK and p38 activation via phosphorylation were observed 8-24h after infection with 0.1 MOI r-MRV in JEG-3 cells showed increase in phosphorylation of p54 and p46 components of JNK and phosphorylation of p38 MAPK as well compared to uninfected vehicle cells. Similarly, JNK and p38 activation via phosphorylation was also observed in JAR cells (B) and HTR-8 (C) 24 h post infection with 0.1 MOI r-MRV compared to uninfected vehicle cells. Total JNK and Total p38 levels were mostly unaltered between ZIKV infected and vehicle uninfected cells. (D) Activation of JNK was also seen with 0.1 MOI PRV 24h-48h post infection in JEG-3 cells compared to uninfected vehicle cells.

Inhibition of JNK prevents ZIKV-induced trophoblast apoptosis: We used small molecule inhibitors of ER stress mediators like selective dephosphorylation inhibitor of eIF2α (Salubrinal, 20μM), IRE1α inhibitor (STF-083010, 20 μM), JNK inhibitor (SP600125, 50 μM), p38MAPK inhibitor (SB203580, 50 μM) or pan caspase inhibitor (Z-VAD-FMK, 50 μM) to test the critical mediators of apoptosis. Similar to figure 2.1, ZIKV infection to trophoblast showed increased apoptosis as evidenced by dramatic increase in caspase 3/7 activity and percent apoptotic nuclei (Fig.2.7). Treatment of ZIKV-infected trophoblast with SP600125 and Z-VAD-fmk showed a significant decrease in caspase 3/7 activation and percent apoptotic nuclei in trophoblasts infected with ZIKV. These results suggest that JNK and caspase activation are critical pathway that drive the ZIKV infected cells with persistent ER stress towards apoptosis (Fig.2.7A-

E). IRE1α inhibition using STF-083010 in JEG-3 cells showed partial protection against ZIKV-induced trophoblast apoptosis, however IRE1α inhibition does not significantly protect against ZIKV-induced apoptosis in HTR-8 cells (Fig.2.7). In contrast, inhibition of ER stress using salubrinal significantly protected against ZIKV-induced apoptosis in HTR-8 cells. Treatment of salubrinal in JEG-3 cells showed a slight non-significant trend in protection against ZIKV-induced apoptosis (Fig.2.7A). In JAR cells, caspase 3/7 activity was significantly inhibited when the infected cells were treated with salubrinal, or SP600125 or Z-VAD-fmk. In contrast, treatment of p38 MAPK inhibitor, SB203580 in ZIKV infected JAR cells showed significantly increased caspase 3/7 activation (Fig.2.7E). These data suggest that activation of caspases and JNK are critical in ZIKV-induced trophoblast apoptosis. Similarly, *XBP1* gene splicing assay showed increased *XBP1* gene splicing in JEG-3 with 0.1MOI r-MRV after 48h post infection compared to uninfected vehicle cells (Fig.2.8A). On the other hand, when the MRV infected cells treated with SP600125 (JNK inhibitor), we observed a dramatic reduction in the levels of XBP1 splicing. Interestingly, treatment of Z-VAD-fmk to the ZIKV infected cells showed dramatic increase in XBP1 splicing and however treatment of salubrinal showed a moderate increase in XBP1 splicing compared to the 0.1rMRV infection alone (Fig2.8A). We then analyzed the expression of viral E protein using immunofluorescence in JEG-3 cells with ZIKV infection. There was a dramatic increase in viral E protein staining in 0.1r-MRV infected JEG-3 cells but was absent in uninfected vehicle cells. The DAPI panel shows fragmented nuclei in the 0.1 rMRV infected cells. ZIKV Infected cells treated with SP600125 (JNK inhibitor) showed dramatic reduction in the viral E protein staining. However, treatment of salubrinal showed moderate reduction

in the viral E protein staining compared to the infected cells. Interestingly, treatment of Z-VAD-fmk in 01.rMRV infected cells showed enhanced viral E protein staining and the nuclei in the DAPI panel are intact confirming the fact that Z-VAD-fmk has inhibited apoptosis suggesting that the viral particles are trapped inside the cells with the inhibition of caspase activity and are protected from viral release for further infection. These data suggests that ZIKV induces ER stress in a JNK-dependent manner and inhibition of JNK prevents ZIKV-induced ER stress and apoptosis.

Figure 7



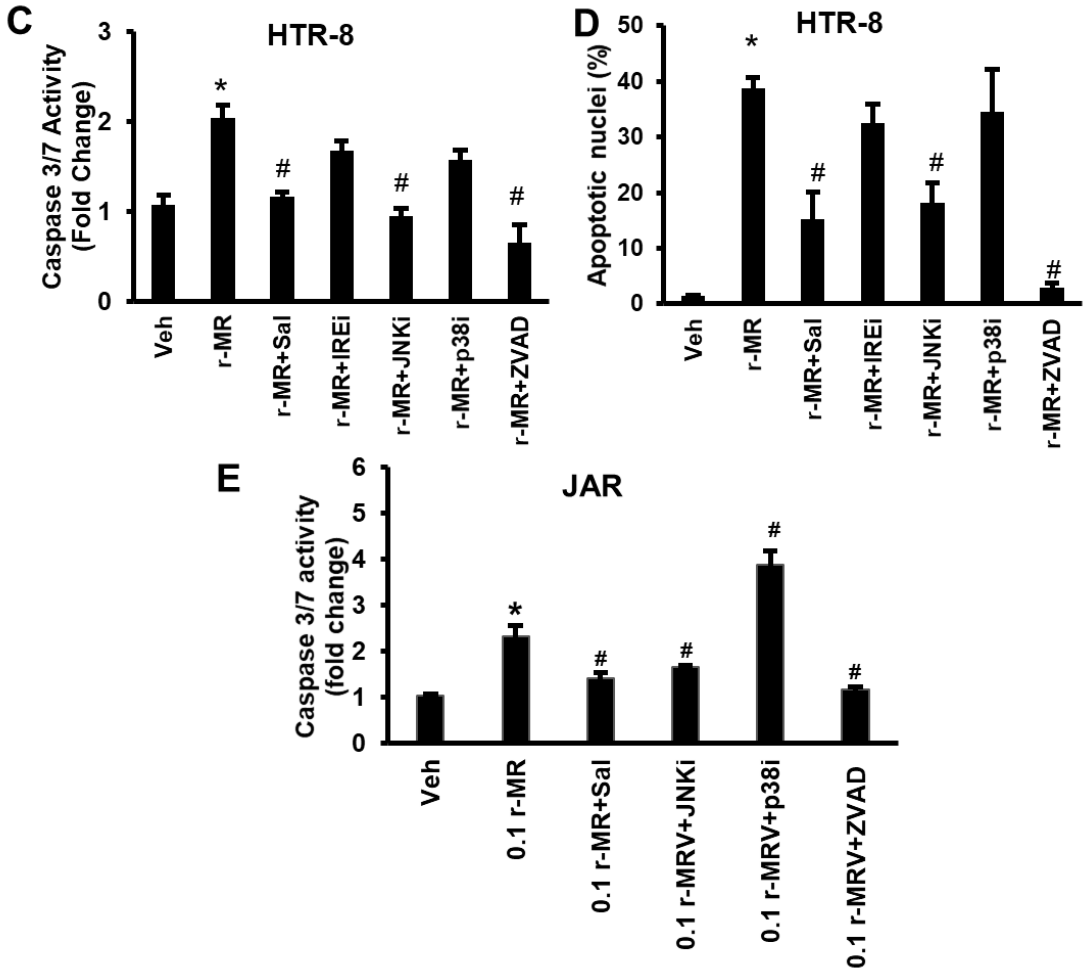
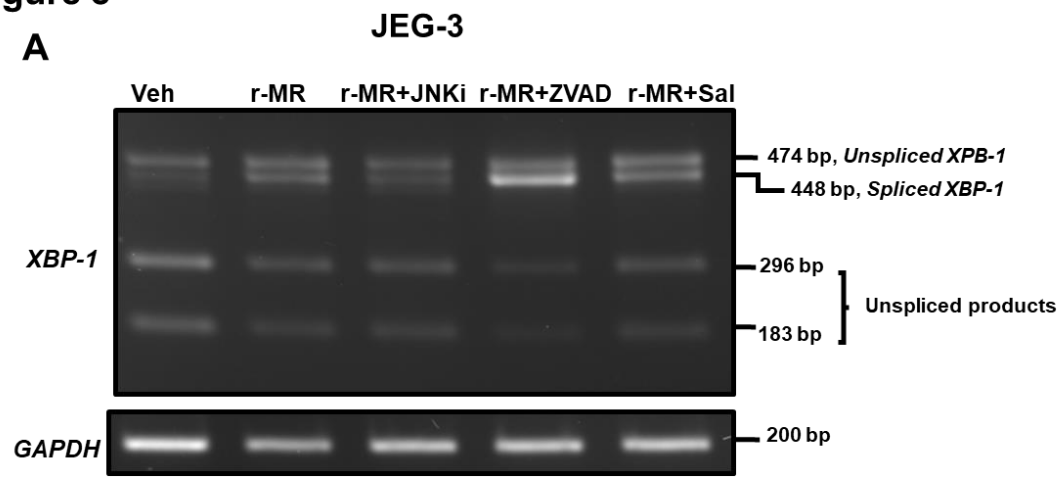


Fig.2.7. Inhibition of JNK prevents ZIKV-induced trophoblast apoptosis.

(A-B) JEG-3 cells infected with 0.1 MOI of r-MRV for 48 h showed significant increase in apoptosis as evidenced by caspase 3/7 activity and percent apoptotic nuclear compared to uninfected vehicle treatment. ZIKV-induced placental trophoblast apoptosis were significantly prevented in JEG-3 cells infected with 0.1 MOI of r-MRV and IREi (STF-083010, IRE1α inhibitor), JNKi (SP600125, JNK inhibitor), or ZVAD (Z-VAD-fmk, pan caspase inhibitor) treatment, respectively. However, ZIKV infection and treatment of Sal (Salubrinal, eIF2α dephosphorylation inhibitor) did not prevent trophoblast apoptosis. (C-D) HTR-8 cells infected with 0.1 MOI of r MRV for 72 h showed a dramatic increase in biochemical markers of apoptosis compared to uninfected vehicle cells. ZIKV-induced placental trophoblast apoptosis were significantly prevented in HTR-8 cells infected with 0.1 MOI of r-MRV and Sal (Salubrinal, eIF2α dephosphorylation inhibitor), JNKi (SP600125, JNK inhibitor), or ZVAD (Z-VAD-fmk, pan caspase inhibitor) treatment, respectively. However, ZIKV infection and IRE inhibition (STF-083010, IRE1α inhibitor) or p38 inhibition treatment does not prevent ZIKV-induced trophoblast apoptosis. (E) JAR cells infected with 0.1 MOI r-MRV 48h post infection showed

activation of caspase 3/7 when compared to vehicle cells. Sal (Salubrinal, eIF2α dephosphorylation inhibitor), JNKi (SP600125, JNK inhibitor), or ZVAD (Z-VAD-fmk, pan caspase inhibitor) treatment in infected cells inhibited caspase 3/7 activation while p38 inhibition significantly increased caspase 3/7 activation. Each value represents mean ± SEM of n=4 for caspase 3/7 activity and n=3 for percent apoptotic nuclei, *P < 0.05 compared to uninfected vehicle cells; #P<0.05 compared to ZIKV infected cells; statistical comparison by ANOVA with post hoc Bonferroni correction.

Figure 8



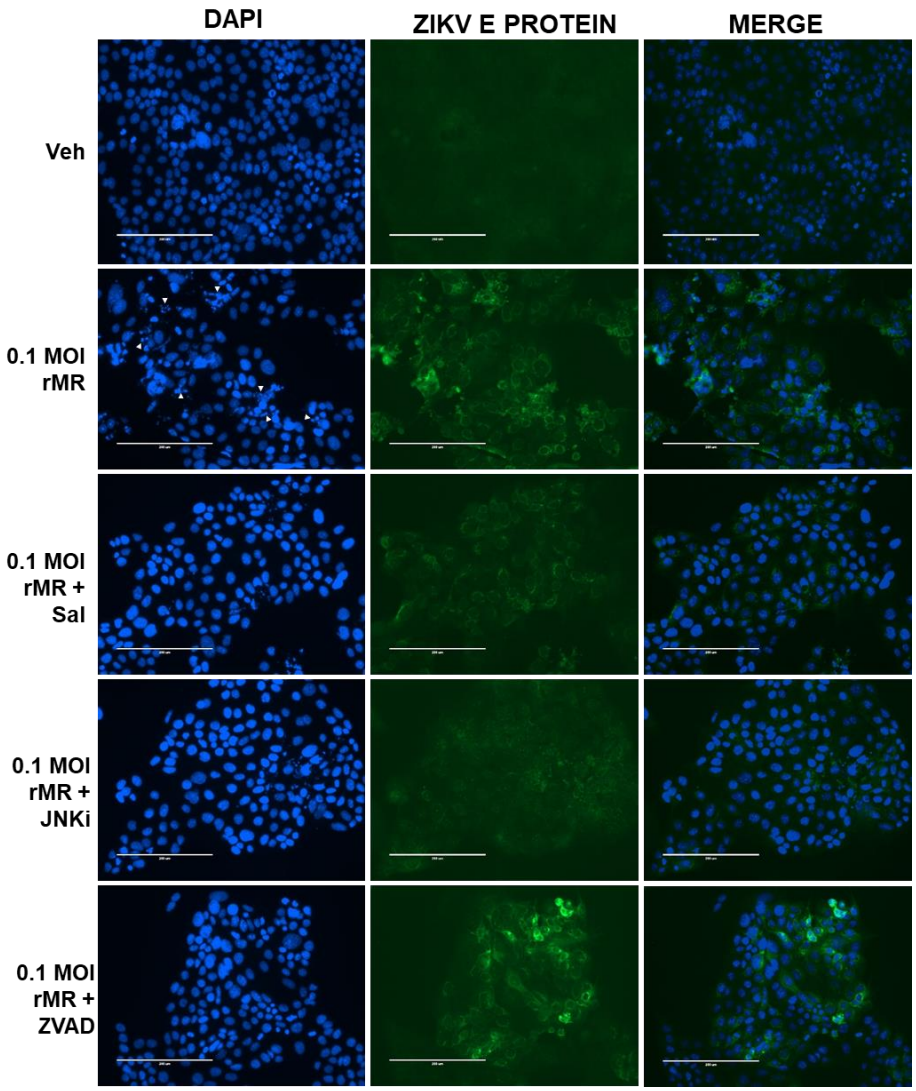


Fig.2.8. Inhibition of JNK reduces XBP1 gene splicing and viral E protein.

(A) JEG-3 cells showed an increase in the splicing of XBP1 gene of the IRE1α arm of ER stress pathway with 0.1 MOI r-MRV, 48h post infection compared to uninfected vehicle cells. XBP1 gene splicing was reduced in the infected cells cotreated with JNKi (SP600125, JNK inhibitor). XBP1 gene splicing were increased in infected cells cotreated with ZVAD (Z-VAD-fmk, pan caspase inhibitor), similarly a moderate increase in XBP1 gene splicing were observed in Sal (Salubrinal, eIF2α dephosphorylation inhibitor) cotreatment to infected cells. (B) JEG-3 cells with 0.1 MOI r-MRV infection also showed viral E protein staining and fragmented nuclei in the DAPI staining compared to the uninfected vehicle cells. Treatment of JNKi (SP600125, JNK inhibitor) to r-MRV infected cells showed reduced E protein staining whereas the ZVAD (Z-VAD-fmk, pan caspase inhibitor) treated infected cells showed huge increase in viral E-protein staining with intact nucleus in the DAPI panel. The Sal (Salubrinal, eIF2α

dephosphorylation inhibitor) treated infected cells also showed moderate viral E protein staining compared to r-MRV infected cells.

2.5 Discussion

The mechanism of ZIKV infection-induced trophoblast apoptosis is via activation of ER stress markers and JNK activation. The principal findings of this manuscript are: 1) ZIKV infection induces caspase dependent apoptosis; 2) ZIKV infection induces three arms of ER stress; 3) Nuclear translocation of CHOP was observed in trophoblasts infected with ZIKV; and 4) Activation of JNK plays a critical role in ZIKV infection-induced trophoblast apoptosis. Even though initially it was thought that term placenta is resistant to ZIKV infection (33) later it was found that ZIKV can cross the placental barrier by compromising the tight junctions (34). Human placenta explant studies have also showed that there was an effective ZIKV replication in the placental tissues and trophoblast apoptosis (35). There are also reports showing ZIKV replication in placental trophoblast cell lines (20,36,37).ZIKV is known to activate innate immune pathways leading to inflammation in placental trophoblasts(20,37). ZIKV infection can also cause placental trophoblasts to undergo apoptosis and release of new viral particles from the dead cells could facilitate ZIKV infection and spread to the growing fetus (38). Interestingly, innate immune pathways can also stimulate apoptosis(39). We also have observed caspase activation and characteristic apoptotic nuclear morphological changes in ZIKV infected trophoblasts, both first and third trimester-derived trophoblasts cells. ZIKV infected human fetal neural parenchymal tissue showed increased expression of Fas and Fas ligand suggesting that apoptosis contributes to fetal brain injury in ZIKV-induced microcephaly (40,41). Our data suggest that there was slight decrease in anti-apoptotic proteins like Bcl2. Recently, ZIKV infection has been shown to delay apoptosis at early

stages of infection to ensure the effective production of virions by regulating Bcl2 (42), this could be attributed to the reason that we could see only subtle changes in the Bcl2 levels after 8h to 24h of infection, a time point where we observed changes in ER stress markers. These data suggest that ER stress precedes apoptotic events with ZIKV infection in trophoblasts. Further time course studies are required to measure the levels of pro-apoptotic and anti-apoptotic proteins with ZIKV infection-induced trophoblast apoptosis.

Flaviviruses are closely associated with ER stress as they replicate within the cellular membrane bound organelles, especially ER. Accumulation of structural and non-structural proteins in the ER results in the formation of convoluted spherules, which activates unfolded protein response (UPR). Apart from this, they also remodel ER in terms of its protein and lipid content (43,44). Zika virus is a positive stranded RNA virus that has been shown to replicate in the ER membrane (44). During viral infection, host cells activates UPR to combat the viral protein load in the ER membranes and induces ER stress and prolonged ER stress result in apoptosis (45,46). Further, ZIKV infection to neural progenitor cells were also shown to upregulate ER stress and apoptosis and could results in the pathophysiology of ZIKV-associated microcephaly (47,48). ER stress activates three different arms of the UPR, namely the PERK pathway, the IRE1α pathway and the ATF6 pathway. There is always crosstalk among these pathways that orchestrates signals leading to survival or death of cells (49).

Sustained ER stress and splicing of *XBP1* gene corresponds well to apoptosis (50,51). IRE1α and XBP1 targets were also shown to be activated in ZIKV infected neuronal cells (52). Although IRE1α and XBP1 activation is important for maintaining

normal protein folding in the ER and biosynthesis of ER (53). We observed increased apoptosis in placental trophoblasts with ZIKV infection along with increased *XBP1* splicing and IRE1α activation suggests that the mechanism of ZIKV-induced apoptosis could be due to the sustained ER stress (50,51).

Sustained ER stress-induced IRE1 α activation has also been shown to activate MAP Kinases like JNK and p38 MAPK via phosphorylation. Sustained or prolonged ER stress is known to activate IRE1α which can result in the IRE1α dependent activation of JNK and p38 MAPK in association of Apoptosis signal-regulating kinase 1 (ASK1) and TNF Receptor Associated Factor 2 (TRAF2) can results in apoptosis (54,55). Further, p38 MAPK is a critical regulator of pro-inflammatory cytokine and is found to be involved in ZIKV induced inflammation of neuronal cells of the retina (56). Further studies are underway to elucidate the critical role for p38 MAPK activation in inflammatory processes in placental trophoblasts with ZIKV infection.

Global protein translation shutdown was observed in ZIKV infected trophoblast as evidenced by enhanced phosphorylation of eIF2α suggesting PERK activation (57). Double-stranded RNA-dependent protein kinase (PKR) also phosphorylates eIF2α which is associated with antiviral interferon signaling pathway to keep viral replication under control (58). Despite the potential to encounter a strong anti-viral interferon response, ZIKV evades the innate immune system by modulating RIG-I (retinoic acid-inducible gene I) like receptor (RLR) signaling, thereby diminishing the effect of anti-viral interferon response to the replicating ZIKV (59). Increased levels of phospho-eIF2α is known to increase the formation of stress granules which composes preinitiation complexes and mRNA for its antiviral activity. However, ZIKV effectively evades this

mechanism and uses the stress granule components to their advantage to aid their own replication (60,61). Bip or GRP78 is a chaperone that binds to the ER membrane proteins IRE1α, PERK and ATF6 in normal resting state, under ER stress it gets dissociated allowing the membrane proteins to be activated (62). PERK phosphorylation leads to ATF4 transcription which in turn results in CHOP nuclear activation (63,64). CHOP primes the cell for apoptosis by decreasing the expression of anti-apoptotic Bcl_2 proteins and increasing expression of GADD45 (65,66). Our data suggests that ZIKV infection induces both CHOP and GADD45 levels in the nucleus. Further, GADD45 is known to be associated with regulation of apoptosis under stress or DNA damage (67). Bip, a master regulator of ER stress is slightly increased 24h post infection in JEG cells but this increase did not occur in JAR and HTR-8 cells with ZIKV infection**.** In a study involving Borna disease virus, Purkinje cells showed CHOP induction without upregulation in the Bip levels. However, astrocytes showed the nuclear activation of CHOP along with Bip upregulation, it was suggested that upregulation of proteins like Bip and protein-disulfide isomerase can protect the cells from apoptosis or delay apoptosis and different cell types responds to ER stress in different ways (68). HERPUD1, an ER resident protein and a component of ERAD pathway has been shown to be upregulated in Enterovirus 71 infections. HERPUD1 upregulation is associated with limiting virus replication by generating interferon mediated immune response (69). It is unclear whether HERPUD1 upregulation is prevented during ZIKV infection in trophoblasts to ensure a productive infection by avoiding any innate immune response. However, further studies are required to confirm role of HERPUD1 in innate immune response evasion by flavivirus infection.

JNK is a master regulator of various physiological processes and are activated in response to stress and can modulate cell survival and cell death pathways (70). Our data show that inhibition of JNK using small molecule led to the discovery that ZIKV-induced trophoblast apoptosis is a JNK-dependent process. JNK activation was also recently reported in mouse brain with ZIKV infection and in dengue virus infected human macrophages (71,72). In our study we found that JNK activation is critical in activating caspases to cause programmed cell death in ZIKV infected placental trophoblasts. JNK activation is also well-established mediator that drives the cells undergoing ER stress into apoptotic pathway. Activation of JNK is known to enhance the translocation of Bax and Bak to the mitochondria, leading to Bax-Bak oligomerization and mitochondrial pore formation, which can result in activation of intrinsic pathway of apoptosis (73,74).

ZIKV-induced trophoblast apoptosis was also protective with different inhibitors of ER stress pathway in a cell specific manner. Inhibition of IRE1α protects against apoptosis in third trimester derived placental trophoblasts. Selective inhibition of eIF2a dephosphorylation using salubrinal protects ZIKV infection-induced first trimester-derived trophoblast apoptosis. However, further studies are required to elucidate the protective role of ER stress inhibitors in suitable animal models of infection at various gestational ages. Expression profile of ZIKV viral entry cofactors varies in the placenta at different stages of gestation, for example, TIM1 is highly expressed in both JEG-3 and HTR-8/SVneo cells but Axl is not expressed in JEG-3 cells (16). Viral entry is a crucial step for replication and there might be a time lag in the replication cycle based on the presence or absence of different viral entry cofactor expression and this could in turn alter the survival dynamics of cells. Even though HTR-8/SVneo cells are derived from

extravillous portion of the first trimester trophoblasts, recent studies show that they contain a population of trophoblasts as well as mesenchymal cells (75) and further HTR-8/SVneo cells undergo epithelial mesenchymal transition which is a characteristic feature in placental development throughout the gestation period (76). Further we also show reduction in *XBP1* gene splicing and viral load with JNK inhibition in ZIKV infected cells linking JNK activation and ER stress pathways. Treatment of salubrinal to ZIKV infected cells caused moderate decrease in *XBP1* gene splicing and viral load, respectively. Interestingly, inhibition of pan-caspase activity caused extensive *XBP1* gene splicing in the infected cells and increased viral load as the viral E proteins are trapped inside the cells and are unable to be released due to inhibition of apoptosis as previous reported (77). A study found that inhibition of autophagy can limit ZIKV infection, this could be correlated to the fact that JNK also has a regulatory control on autophagy pathway and studies have shown that JNK inhibition also causes inhibit autophagy (78-80).

2.6 Conclusion

In conclusion, our data suggest that ZIKV infection induces ER stress and placental trophoblast apoptosis. Mechanistically, we have identified that JNK is a critical mediator of ZIKV-induced trophoblast apoptosis. Further studies are required to identify the molecular mechanism of JNK activation and other interacting partners with ZIKV replication. Trophoblasts are the major cell type that interacts with maternal decidua and maternal blood circulation. Protective strategies against ZIKV infection in trophoblast can be crucial in preventing the transmission of ZIKV from the mother to the fetus.

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CHAPTER 3: PALMITOLEATE PROTECTS AGAINST ZIKA VIRUS-INDUCED PLACENTAL TROPHOBLAST APOPTOSIS

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3.1 Abstract

Zika virus (ZIKV) infection in pregnant women is associated with the development of microcephaly, intrauterine growth restriction, and ocular damage in the fetus. ZIKV infection of the placenta plays a crucial role in the vertical transmission from the maternal circulation to the fetus. Our previous studies suggested that ZIKV induces endoplasmic reticulum (ER) stress and apoptosis of placental trophoblasts. Here, we showed that palmitoleate, an omega-7 monounsaturated fatty acid, prevents ZIKV-induced ER stress and apoptosis in placental trophoblasts. Human trophoblast cell lines (JEG-3 and JAR) and normal immortalized trophoblasts (HTR-8) were used. We observed that ZIKV infection of the trophoblasts resulted in apoptosis and treatment of the infected cells with palmitoleate significantly prevented apoptosis. However, palmitate (saturated fatty acid) did not offer protection from ZIKV-induced ER stress and apoptosis. We also observed that the Zika viral RNA copies were decreased, and the cell viability improved in ZIKV-infected cells treated with palmitoleate as compared to the infected cells without palmitoleate treatment. Further, palmitoleate was shown to protect against ZIKV-induced upregulation of ER stress markers, C/EBP homologous protein and X-box binding protein 1 splicing in placental trophoblasts. In conclusion, our studies suggest that

palmitoleate protects placental trophoblasts against ZIKV-induced ER stress and apoptosis.

3.2 Introduction

Zika virus (ZIKV) was originally identified in the Zika forest of Uganda from the blood sample of febrile macaque (1). ZIKV infection of humans was first reported to occur sporadically in the African continent in early 1960's, but later ZIKV infection spread throughout the world including Yap islands, Pacific islands and the American continent between 2007 and 2016 (2). ZIKV is an arbovirus belongs to the *Flaviviridae* family and is closely related to dengue, chikungunya, and Yellow fever viruses (3). ZIKV usually causes self-limiting disease in a healthy individual, but infection during pregnancy often causes devastating effects to the fetus. ZIKV infected fetus exhibits Congenital Zika syndrome, which is an array of disease manifestations that includes microcephaly, retinal defects, and muscular-skeletal defects (4). Further, ZIKV infection is also associated with the development Guillain-Barre syndrome in adults (5).

Placental route plays a major role in ZIKV transmission from the infected mother to the fetus. Studies suggest that placental trophoblasts, endothelial cells and Hofbauer cells can be infected and plays a crucial role in ZIKV dissemination from mother to fetus (6-9). ZIKV is also known to cause placental dysfunction by driving an inflammatory condition accompanied by apoptosis (8,10,11). Further, ZIKV infection of the placental cells was shown to compromise the normal physiological role of the placenta, which is crucial for the fetal growth and survival (12-14). ZIKV can also cause drastic changes in the placental lipid metabolism affecting the nourishment of growing fetus (15).

There are several candidate ZIKV vaccine which include DNA vaccines, mRNA vaccine and whole live-attenuated virus or inactivated virus vaccines (16-19). Although several candidate vaccines progressed to early clinical trials, they are posed with serious challenges such as case detection after vaccination and vaccination strategies primarily focusing on females who are already pregnant or expecting pregnancy. Similarly, several drug candidates such as niclosamide (anti-parasite drug) and emircasin (pan-caspase inhibitor) to prevent ZIKV infection have been identified (20). However, the safety and administration of niclosamide during pregnancy is under debate (a pregnancy category B drug) and emricasin efficacy is solely attributed to its neuroprotective property rather than its anti-viral activity and has not been tested in pregnant women (21). Similarly, sofosbuvir, a FDA approved nucleotide analog and an inhibitor of NS5B polymerase of Hepatitis C virus has also been shown to be effective against ZIKV infected 3 day old Swiss mice model, but still this a pregnancy category B drug which does not have well-documented studies in humans (22). With these limitations in hand, in the present study, we tested the novel protective role of palmitoleate against ZIKV-induced trophoblast apoptosis. Palmitoleate (16:1 n-7) is an omega-7¸ monounsaturated fatty acid and has been shown to protect against saturated free fatty acid-induced apoptosis of hepatocytes, pancreatic beta cells, and umbilical vein endothelial cells (23-25). This led to our hypothesis that palmitoleate prevents ZIKV-induced ER stress and apoptosis in trophoblasts. Our results reveal that palmitoleate protects trophoblasts from ZIKV-induced ER stress and apoptosis by downregulating the levels of ER stress markers such as CEBP homologous protein (CHOP) and spliced X-box binding protein (XBP1).

3.3 Materials and Methods

Materials: Palmitoleate (#P9417), palmitate (#P5585), fatty acid free BSA (A3803), Steriflip vacuum (#SCGP00525), 4', 6-diamidine-2-phenylindole dihydrochloride (DAPI) (#D9542), and anti-flavivirus group antigen antibody (D1-4G2-4-15 clone) (# MAB 10216) were purchased from MilliporeSigma, MO ,USA. Apo-ONE homogenous caspase 3/7 assay (WI # G7791) was obtained from Promega, Madison, WI, USA. Alexaflour-488 conjugated anti-mouse antibody (#A11001), Trizol Reagent (# 15596018), Superscript II reverse transcriptase, (#18064-014), RNAse out (#10777-019), Random Hexamers (#8080127) were obtained from Thermo Scientific, MA and QIAamp viral-RNA isolation kit was from Qiagen, Hilden, Germany (#52906). Hydrolysis probe (Taqman) for Viral E protein detection were custom synthesized by IDT, IA, USA. Restriction enzyme, Pst I was from New England Biolabs, Ipswich, MA, USA (MA #R0140).

Cells: JEG-3 and JAR cells, human choriocarcinoma-derived third trimester trophoblast cell lines, HTR-8SV/neo (HTR-8), a first trimester human immortalized trophoblasts, and Vero cells were used. MEM for JEG-3 cells and DMEM for JAR and HTR-8 cells containing 10% fetal bovine serum and 0.01 % plasmocin was used for. Vero cells were used for plaque assay and cultured in DMEM (Gibco, Waltham, MA, USA) containing, sodium bicarbonate (3.7g/L), 1X penicillin and streptomycin, 10% FBS, and 0.01% plasmocin. All the cells used in the present study were obtained from ATCC and periodically tested for mycoplasma.

Virus and infection of the trophoblasts: Original Ugandan MR766 strain (MRV) or recombinant MR strain of Zika virus (r-MRV) (26) or PRVABC59 (PRV, Asian lineage isolated from Puerto Rico) were used to infect the cells with 0.1 -1 MOI. Virus infection media containing 2X DMEM (Gibco), 2% fetal bovine serum,100 µg/mL streptomycin, 100 I.U./mL penicillin, 7.5% sodium bicarbonate, 20mM HEPES, sodium pyruvate, 1X nonessential amino acids and 0.01% plasmocin was used. After virus adsorption, the media was replaced with DMEM or MEM containing 10% fetal bovine serum and 1% BSA.

Treatment of fatty acids: Palmitoleate and palmitate were dissolved in isopropanol with a stock solution concentration of 80 mM. Fatty acid-free BSA (1%) was dissolved in growth media at room temperature using a tube rotator and incubated at 37^{o}C for 30 minutes in a water bath and then filter sterilized. Fatty acids were then incubated in the freshly prepared 1% fatty acid-free BSA for fatty acid-BSA conjugation by incubating at 37^{o}C in the water bath for 20 minutes. We have used 100-200 µM concentrations of fatty acids for 48-96 hpi.

Biochemical and structural characterization of apoptosis: Structural and biochemical markers of apoptosis like percent apoptotic nulcei and caspase 3/7 activity, respectively were assessed. Percent apoptotic nuclei was quantified by characteristic nuclear morphology and visualized by treatment with the fluorescent DNA-binding dye, DAPI as described (27). Briefly, cells were stained with 5 µg/mL of DAPI for 5-10 minutes at 37°C. Apoptotic nuclei (condensed, fragmented) were counted and presented as a percent of total nuclei. At least 100 cells were counted per well and experiments were performed in triplicate. Caspase 3/7 activity was measured using rhodamine 110 bis-(N-CBZ-L-

aspartyl-L-glutamyl-L-Valyl-aspartic acid amide (Z-DEVD-R110) substrate. The caspase 3 and 7 enzyme activity in the cells will cleave the DEVD peptide in the substrate and release the rhodamine 110 fluorophore, which can be measured spectrofluorometrically (Biotek Synergy, Winooski, VT, USA) with 498nm wavelength of excitation and 521nm emission. The data were reported as fold-change of net fluorescence compared to vehicle treated cells, with experiments performed in triplicate or quadruplicate.

Immunofluorescence analysis: After 48h of ZIKV infection, the media was aspirated and the cells were washed with phosphate buffered saline (PBS). Fixing was done with methanol and acetone at the ratio of 1:1 and washed thrice with PBS. Primary antibody, anti-flavivirus group antigen antibody (D1-4G2-4-15 clone) was used at a dilution of 1:500 and incubated at room temperature for 2h with gentle rocking. After primary antibody incubation, cells were washed thrice with PBS in 5 minutes intervals. Alexaflour-488 conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) was added at a dilution of 1:1000 and kept in a shaker at room temperature for 1h. After incubation the cells were washed thrice in PBS and then visualized under Nikon A1R-Ti2 confocal system.

Quantitative real time Polymerase Chain Reaction: Total RNA was extracted from cells, 48-96 hpi using TRIzol reagent as described in the manufacturer protocol. Around 1-5µg RNA from each sample was reverse transcribed to cDNA using random hexamers, RNAse OUT and Superscript II. Relative CHOP mRNA expression was quantified using Light cycler 480 SYBR Green I Master Version 13 (Roche, Basel, Switzerland) in a Bio-Rad CFX connect Real – Time System (Hercules, CA, USA). CHOP published primers

as described (28) was used. Housekeeping gene 18S rRNA was used as control and the primer used are listed in table 3.1.

Table 3.1 Primer details

Primer	Forward primer	Reverse primer	Product length
XBP1	5'AAACAGAGTAGCAGCTCAGACTGC 3'	5'TCCTTCTGGGTAGACCTCTGGGAG 3'	unspliced forms ~474bp cleaved by the restriction endonuclease (PstI) - two products are around 296bp and 183bp are formed spliced forms lack restriction enzyme site ~ 448bp
GAPDH	5'AATCCCATCACCATCTTCCA 3'	5'TTCACACCCATGACG AAC AT 3'	~194bp
18srRNA	5'CGTTCTTAGTTGGTGGAGCG 3'	5'CGCTGAGCCAGT CAG TGTAG 3'	~212bp
CHOP	5′-ATGGCAGCTGAGTCATTGCCTTTC-3′	5′-AGAAGCAGGGTCAAGAGTGGTGAA-3	~265bp
Viral Envelope	Forward 5′-GTCGTTGCCCAACACAAG-3′ Reverse 5′-CCACTAATGTTCTTTTGCAGAC-3′		
Hydrolysis probe used for *viral E gene*	5′-/56-FAM (5′ 6-carboxyfluorescein)/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ (3′ Iowa black fluorescent quencher)/-3′		

Quantification of viral RNA copy number using hydrolysis probe: RNA form cell culture supernatant was isolated by QIAamp viral-RNA extraction kit. RNA from cell lysate was isolated by TriZOL and cDNA synthesis was performed. Taqman hydrolysis

probes for quantitative PCR primers targeting E gene of the ZIKV was used (Table 3.1). Absolute RNA quantification was performed using a standard curve generated from PCR product using primers listed in table 3.1 and as described (6).

***XBP1* mRNA splicing assay:**

The cDNA samples with 1:3 or 1:10 dilution was subjected to PCR to amplify *XBP1* gene using the primer set (each 20µM) as described (29). The PCR product (around 8 µL) was digested with 1µL of PstI (20 U) in 1 µL of 3.1 NEB buffer containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/mL BSA pH 7.9 and incubated at 37^{o}C for 2h. The restriction enzyme digested PCR product was electrophoresed in 2% agarose gel stained with ethidium bromide. The unspliced 474 bp nucleotide and can be cleaved by PstI enzyme by recognition of the intact restriction site, resulting in 296 bp and 183 bp fragments. The spliced forms lacks the intact restriction enzyme site so the bands are visualized around 448bp. GAPDH was used as a control and was amplified using primers listed in table 3.1 (30). Relative band intensities were analyzed using Image J software.

Western blot: Cell lysates were scrapped using 100µL of lysis buffer made of 50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM DTT, 1mM Na_3Vo_4, 1mM PMSF, 100mM NaF and 1% Triton x-100 . Cell supernatant obtained after 10,000 x g for 10 min of centrifugation and was used for protein estimation using modified Lowry method-Pierce 660nm protein assay reagent (ThermoFisher Scientific). Around 30µg protein was electrophoresed in a 10% SDS-polyacrylamide gel and transferred into nitrocellulose membrane. The membrane was blocked with 5% BSA in TBST. Primary antibody was

used in 1:1000 dilution in with 5% BSA in TBST. Secondary antibody was used in 1:5000 dilution. Washes of 10 mins each for 3 times were employed after both primary and secondary antibody incubation. The blot was developed using Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA).

Plaque assay: The plaque assay was performed as described (31). Briefly, cell culture supernatants were collected after 48 h of ZIKV infection and fatty acid treatment (48hpi) and diluted serially 10^{-3} to 10^{-4} using virus infection media in duplicates. The diluted supernatant samples were kept for virus adsorption for 1 h over 90% confluent Vero cells and the cells were washed with PBS prior to virus adsorption. After virus adsorption 1:1 ratio of 2% low melting agarose and plaque assay media containing 2x DMEM, 1X penicillin and streptomycin, 4% FBS, sodium bicarbonate 7.5%, 20mM HEPES, sodium pyruvate, 1X nonessential amino acids and 0.01% plasmocin were added to each well and incubated at 37^{o}C for 4 days. Fixation of cells was done by using 10% Formalin in PBS for 1h. After the removal of agarose overlaid in each well, fixed cells were stained with 0.1% crystal violet staining solution for 1h followed by washing the plates with distilled water and allowed to air dry. Plaques were counted in each well and expressed plaque forming units/mL (pfu/mL).

Percent Cell Survival using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): Stock solution containing 50mg of MTT in 10 mL of PBS was filter sterilized and stored at 4^{o}C. Around 20 µL of MTT stock was diluted to 100 in MEM media without any FBS. Media from the cells grown in a 96 well plate was aspirated and then 110 µL of the prepared MTT solution was added to each well. After 4h incubation at 37^{o}C, media was aspirated and 100µL of isopropanol containing 1 µL 37% HCl per 10

mL of isopropanol was added to each well. The contents were gently mixed and the absorbance was measured at 540 nm.

Assessment of cell viability using crystal violet: The assay was performed as described by (32). Briefly, In a 24 well plate 30,000 cells were seeded and infected with ZIKV and fatty acids were treated after 1 h of virus adsorption. After 48h, media was aspirated and washed two times with double distilled water. The cells were then stained with 300µL of 0.5% crystal violet staining solution per well for 20 mins at room temperature with gentle rocking. The excess stain after incubation was washed four times with double distilled water and air dried for 2h by inverting the plate onto a blotting paper. Once the plate was dried, around 500of methanol was added to each well and placed on a rocker for 20 mins at room temperature and the absorbance was measured at 570nm.

2.14. Data analysis: Data are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using Welch's t test, P value <0.05 was considered as statistically significant.

3.4 Results

Palmitoleate prevents ZIKV-induced placental trophoblast apoptosis: We observed the characteristics apoptotic nuclear morphological changes in ZIKV-infected placental trophoblasts, and showed a dramatic increase in percent apoptotic nuclei after 1.0 MOI of PRV infection for 48-96 h in JEG-3 cells (Fig.3.1A, B, and Supplementary Fig.1A). Percent apoptotic nuclei was significantly reduced with the treatment of 100-200µM of palmitoleate after 1 MOI of PRV infection for 48-96h in JEG-3 cells compared ZIKV infection alone (Fig.3.1A, B, Supplementary Fig.1A). Similarly, treatment of ZIKV

infected trophoblasts with palmitoleate significantly downregulated caspase 3/7 activity after 48-72 h of infection compared PRV infection alone in JEG-3 cells (Fig.3.1A, B).

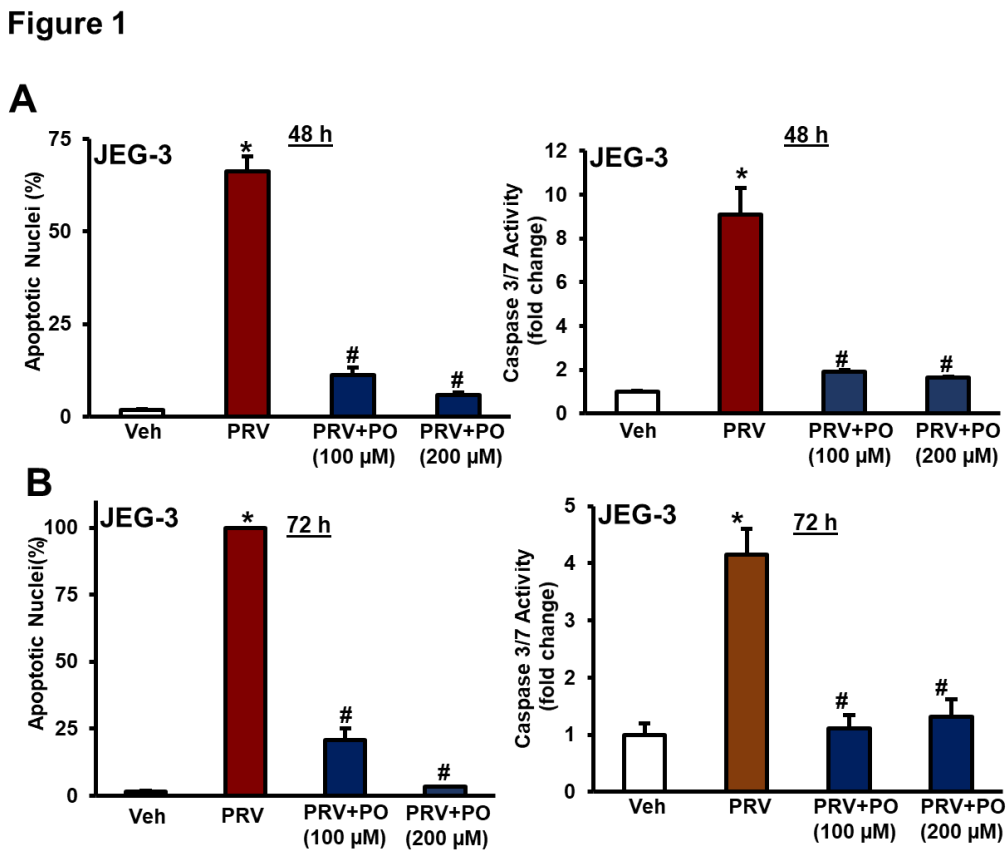
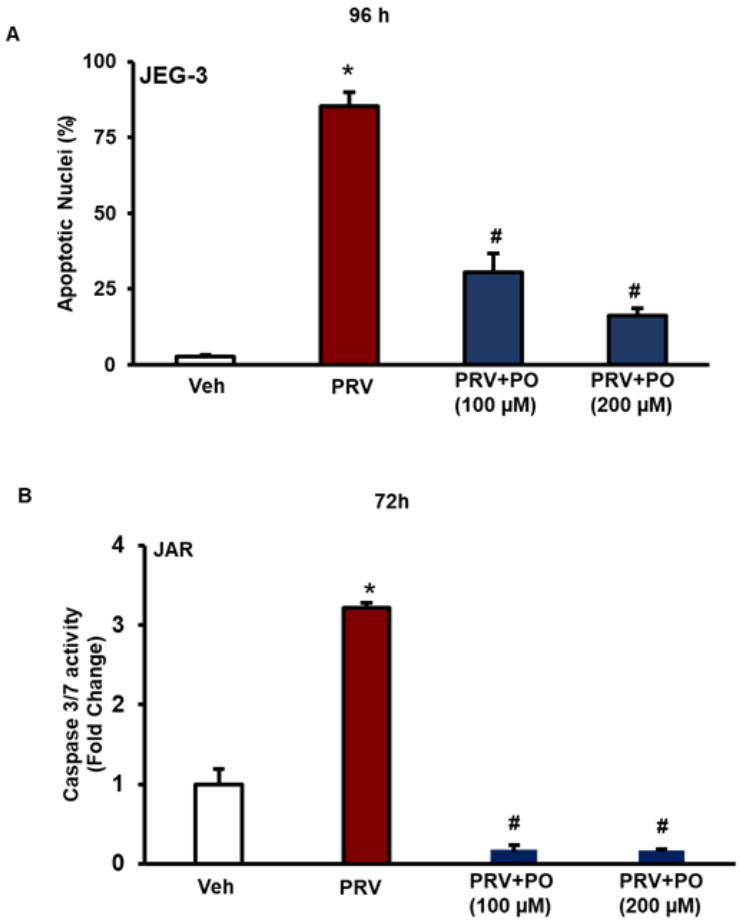


Fig.3.1. Palmitoleate treatment after Zika virus (ZIKV) PRVABC59 strain (PRV) infection inhibits apoptosis in JEG-3 cells.

(A) Structural and biochemical characterization of apoptosis in JEG-3 infected with 1.0 MOI (multiplicity of infection) ZIKV PRV strain, 48-72 h showed increase in percent apoptotic nuclei compared to uninfected vehicle cells. Infected cells treated with 100µM or 200µM of palmitoleate, 48-72hpi (hours post infection) showed a decrease in the percent apoptotic nuclei when compared to ZIKV infected cells alone (left panels). (B) There was a significant activation of caspase 3/7 in ZIKV infected cells, but caspase 3/7 activation was significantly blocked when the infected cells were treated with 100 or 200µM of palmitoleate both at 48 and 72 hpi (right panels). Data represents mean ±SEM, n=3.*P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.



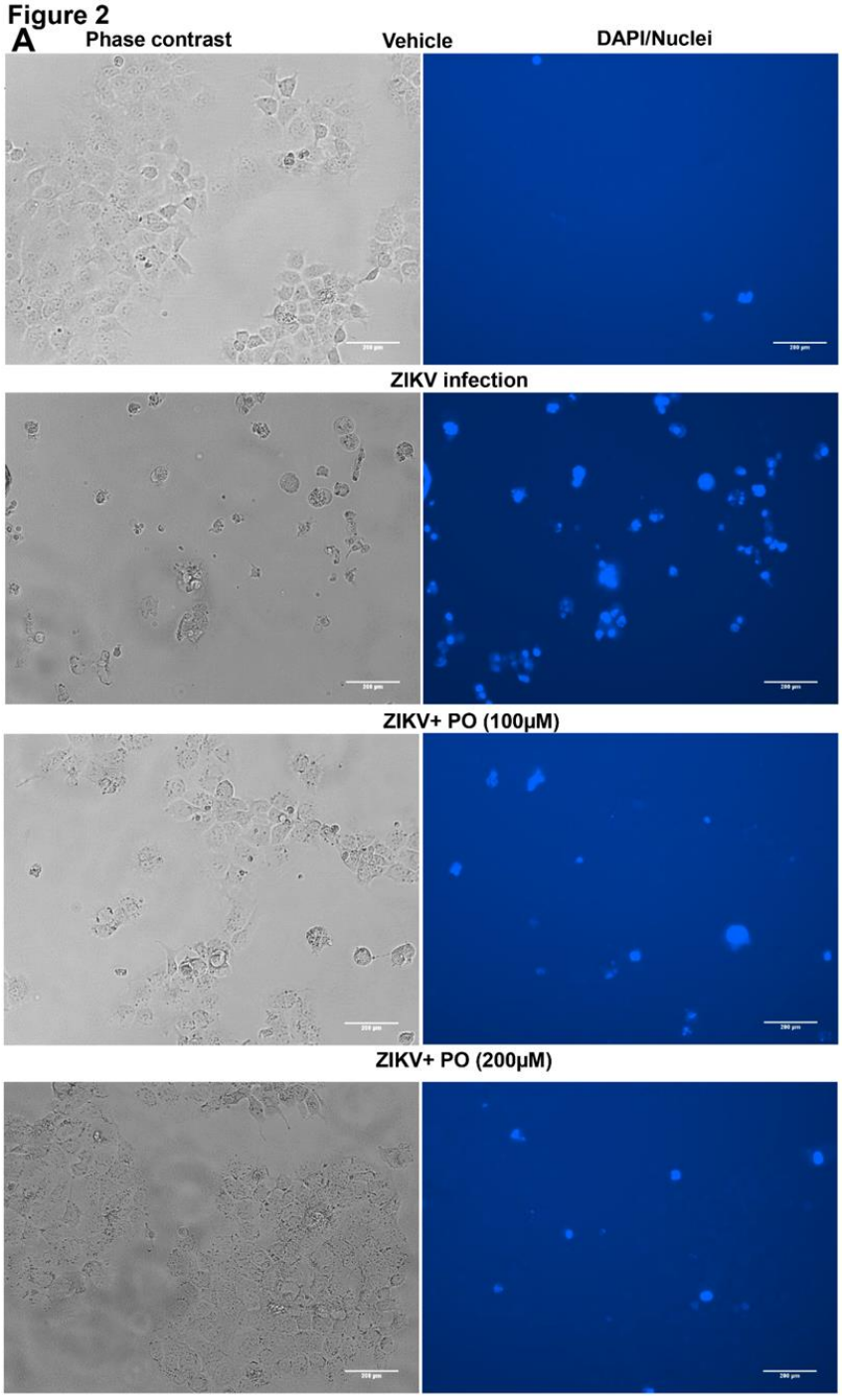
Supplementary Fig.1. (A) Increase in percentage apoptotic nuclei in JEG-3 cells infected with 1.0 MOI PRV 96hpi when compared to uninfected vehicle cells. On treatment with 100-200 µM palmitoleate post infection there was significant reduction in the percent apoptotic nuclei. (B) Similarly, there was significant upregulation of caspase 3/7 in 1.0MOI PRV infected JAR cells when compared to uninfected vehicle cells but this was significantly downregulated when the cells were treated with 100-200 µM palmitoleate post infection. Data represents mean ±SEM, n=3.*P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.

We next tested the protective role of palmitoleate in another human term-derived placental trophoblast (JAR cells) using MRV, original Ugandan strain for 48h. JAR cells infected with MRV showed dramatic increase in the number of cells that show fragmented and condensed nuclei compared to vehicle cells. Increased nuclear morphological changes were prevented with the treatment of palmitoleate in trophoblasts

(100-200 µM, Fig.3.2A). Further, increased percent apoptotic nuclei observed in 1.0 MOI MRV infection was dramatically reduced with the treatment of 100 and 200µM palmitoleate in JAR cells (Fig.3.2 A-B). We also observed a significant decrease in caspase 3/7 activity with 200 µM palmitoleate treatment and a trend in decreased caspase 3/7 activity with the 100 µM palmitoleate treatment in 1.0 MOI MRV infected trophoblasts (Fig.3.2C). Similarly, JAR cells infected with 1.0 MOI of PRV also showed increased caspase 3/7 activity and this was prevented with the treatment of 100-200 µM palmitoleate (Supplementary Fig. 1B).

To test whether palmitoleate would also protect against first-trimester derived placental trophoblast cells, we used HTR-8 cells. Similar to JEG-3 and JAR cells, HTR-8 cells showed enhanced apoptosis as evidenced by an increase in the levels of percent apoptotic nuclei and caspase 3/7 activity with 1.0 MOI of r-MRV for 72h (Fig.3.2D). Treatment of 100 and 200µM palmitoleate to 1.0 MOI of r-MRV infected HTR-8 cells significantly reduced the percent apoptotic nuclei and caspase 3/7 activation (Fig.3.2E).

Figure 2


A
Phase contrast
Vehicle
DAPI/Nuclei
ZIKV infection
ZIKV+ PO (100μM)
ZIKV+ PO (200μM)

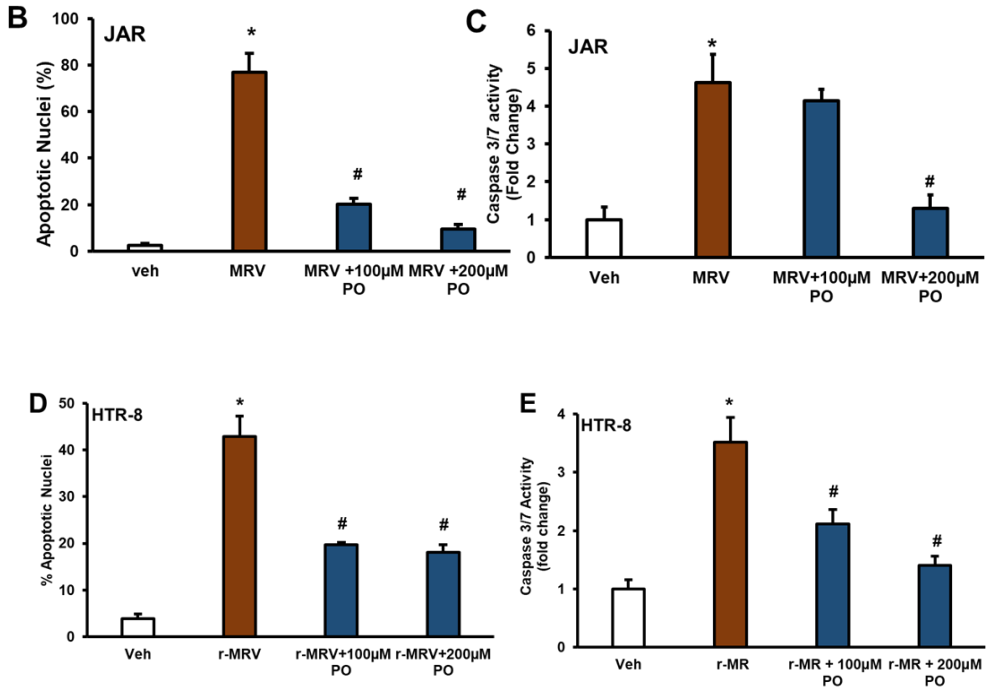


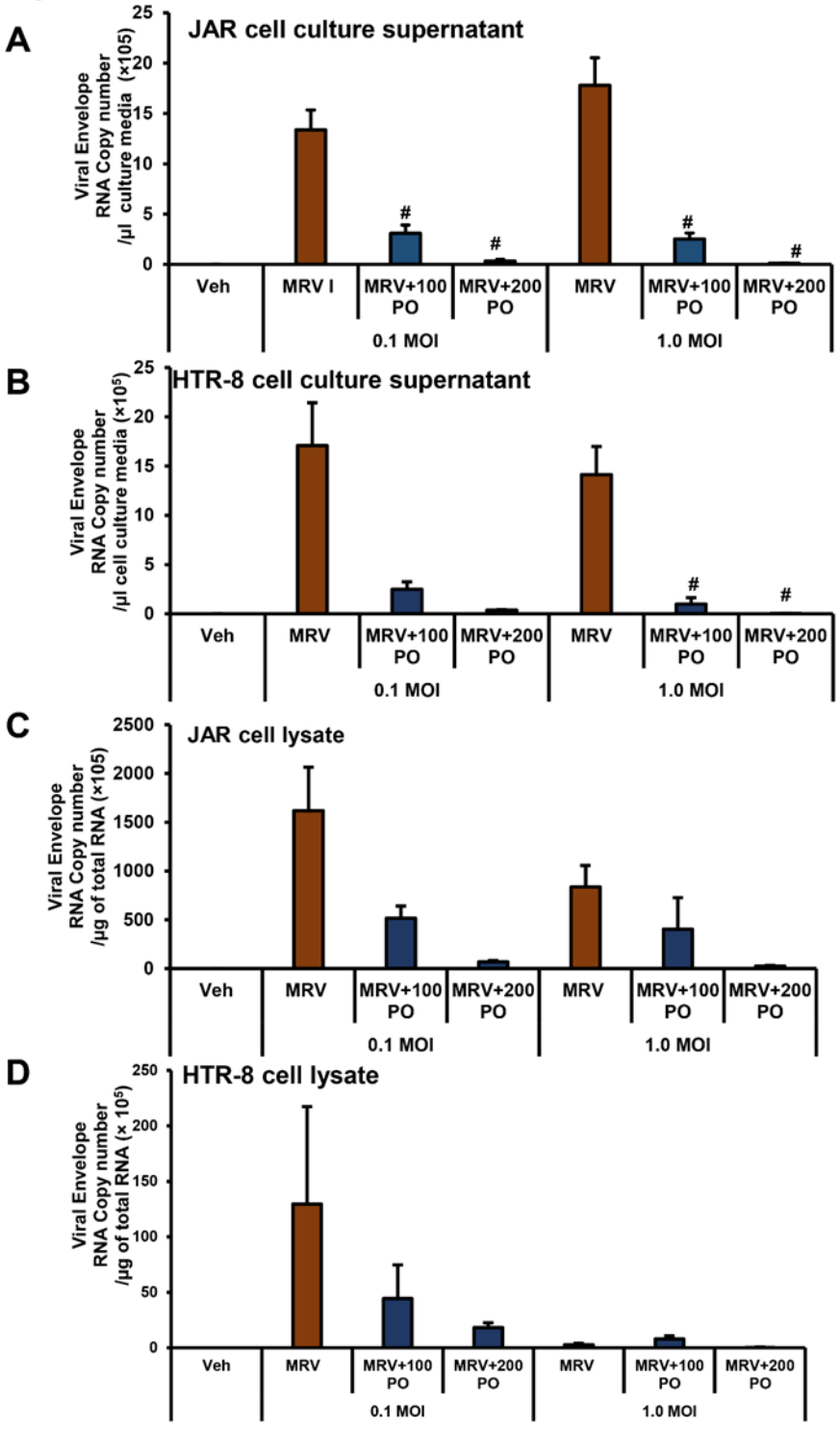
Fig.3.2. Treatment of the placental cells with palmitoleate after MR766 strain of ZIKV infection inhibits apoptosis.

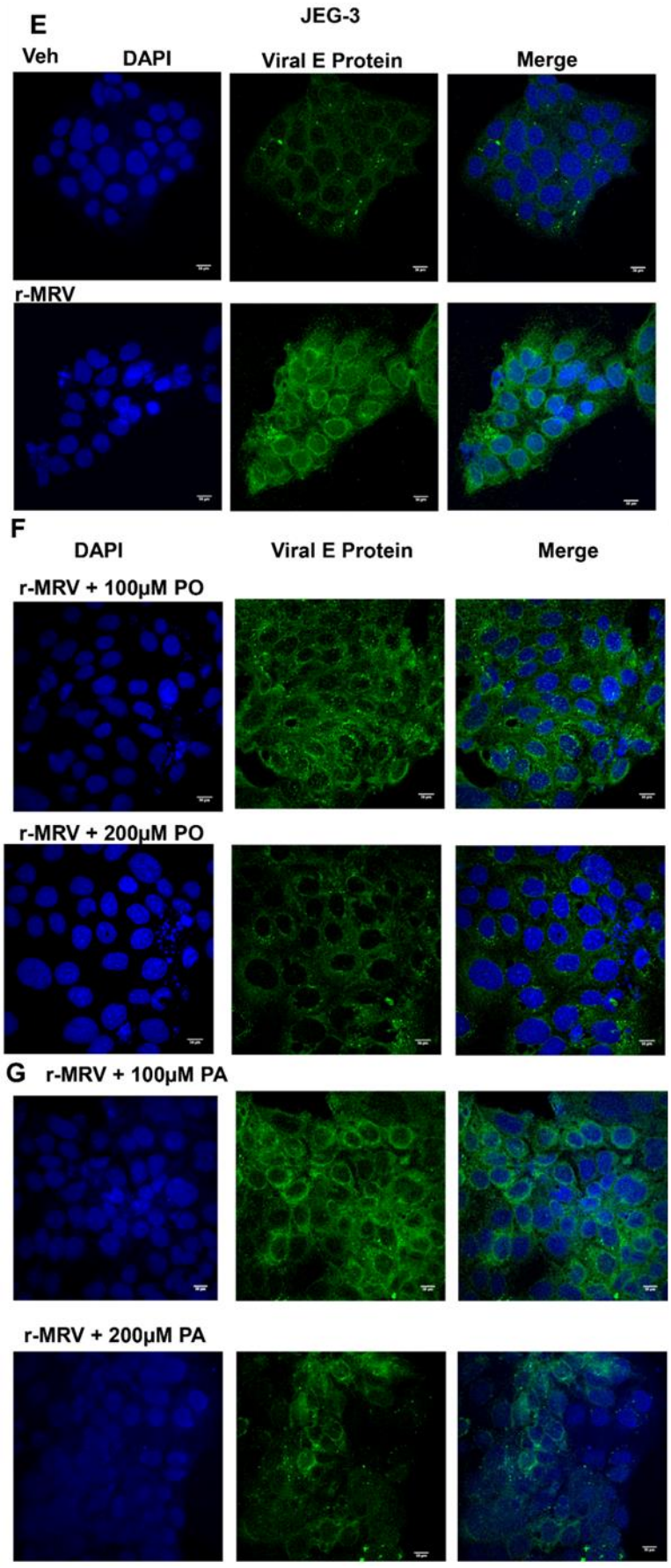
(A) Phase contrast (left panels) and DAPI stained (right panels) image panels: Characteristic nuclear morphological changes were detected with DAPI staining in third trimester-derived JAR cells after 48 hpi with MRV. Vehicle cells show little to no DAPI staining. Infected panel shows higher number of DAPI stained fragmented and condensed nuclei compared to 100 or 200µM pamitoleate treated cells. The images shown here are representative images. (B) JAR cells infected with 1.0 MOI MR strain of ZIKV showed increase in the percentage apoptotic nuclei when compared to uninfected vehicle cells. In cells treated with 100µM and 200µM palmitoleate 48 hpi showed a significant decrease in the percent apoptotic nuclei compared to ZIKV infected cells. (C) There was a significant increase in caspase 3/7 activity in MRV infected cells, but this was significantly blocked when the cells were treated with 200µM of palmitoleate post infection and a trend towards downregulation at 100µM pamitoleate treatment in the MRV infected cells. (D) Characteristic nuclear morphological changes were detected using DAPI staining in first trimester derived HTR-8 cells after 72 hpi were calculated and represented as percent apoptotic nuclei. Cells infected with 1.0 MOI r-MR strain of ZIKV showed increase in the percent apoptotic nuclei when compared to uninfected vehicle cells. However, cells treated with 100µM and 200µM palmitoleate post infection showed a significant decrease in the percentage apoptotic nuclei compared to ZIKV-infected cells. (E) There was an increase in caspase 3/7 activity in infected cells when compared to uninfected vehicle cells but this activation was significantly blocked when the cells were treated with both 100 and 200 µM of palmitoleate, 72 hpi. Data represents

mean ±SEM, n=3 for percent apoptotic nuclei and n=4 for caspase 3/7 activity.*P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.

Treatment of palmitoleate to ZIKV infected trophoblasts reduces viral RNA copy number: JAR cells were infected with MRV for 72 h and palmitoleate treatment (100 and 200µM) showed a dramatic decrease in the Zika viral (E gene) RNA copy number in cell culture supernatant with both 0.1 and 1 MOI and a trend towards decrease in cell lysate (Fig.3.3A and 3.3C). This suggests that palmitoleate interferes with ZIKV replication and its release from infected placental trophoblasts. Similarly, HTR-8 cells infected with MRV for 96h showed significant reduction in viral envelope RNA copy numbers with the treatment of palmitoleate (100 and 200µM) with 1 MOI infection and a trend towards reduction with 0.1 MOI in cell culture supernatant (Fig.3.3B). Palmitoleate treatment with both 0.1 and 1 MOI in HTR-8 cells showed only a trend towards reduction in viral RNA copy number in cell lysate (Fig.3.3D).

Figure 3


A
JAR cell culture supernatant
Viral Envelope RNA Copy number /µl culture media (×105)
25
20
15
10
5
0
#
#
#
Veh
MRV I
MRV+100 PO
MRV+200 PO
MRV
MRV+100 PO
MRV+200 PO
0.1 MOI
1.0 MOI
B
HTR-8 cell culture supernatant
Viral Envelope RNA Copy number /µl cell culture media (×10⁵)
25
20
15
10
5
0
#
#
Veh
MRV
MRV+100 PO
MRV+200 PO
MRV
MRV+100 PO
MRV+200 PO
0.1 MOI
1.0 MOI
C
JAR cell lysate
Viral Envelope RNA Copy number /µg of total RNA (×105)
2500
2000
1500
1000
500
0
Veh
MRV
MRV+100 PO
MRV+200 PO
MRV
MRV+100 PO
MRV+200 PO
0.1 MOI
1.0 MOI
D
HTR-8 cell lysate
Viral Envelope RNA Copy number /µg of total RNA (× 10⁵)
250
200
150
100
50
0
Veh
MRV
MRV+100 PO
MRV+200 PO
MRV
MRV+100 PO
MRV+200 PO
0.1 MOI
1.0 MOI


E
JEG-3
Veh
DAPI
Viral E Protein
Merge
r-MRV
F
DAPI
Viral E Protein
Merge
r-MRV + 100μM PO
r-MRV + 200μM PO
G
r-MRV + 100μM PA
r-MRV + 200μM PA

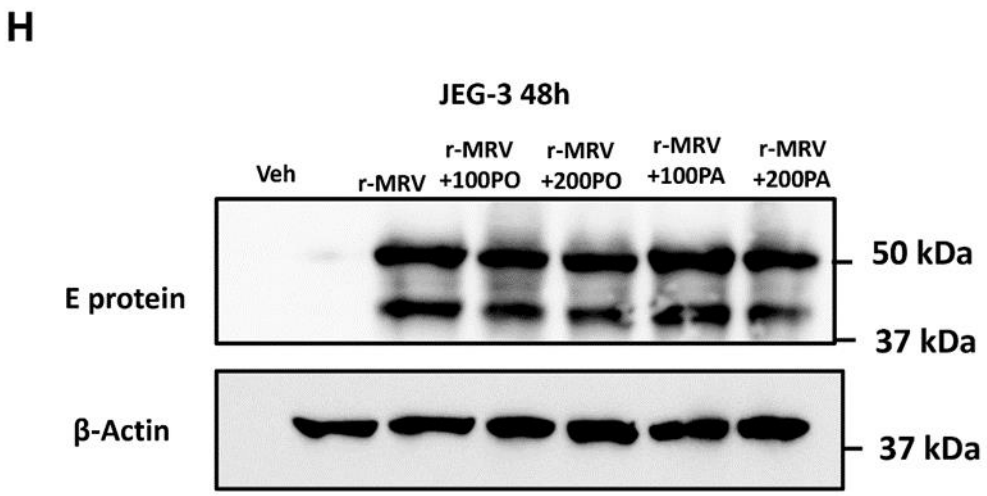


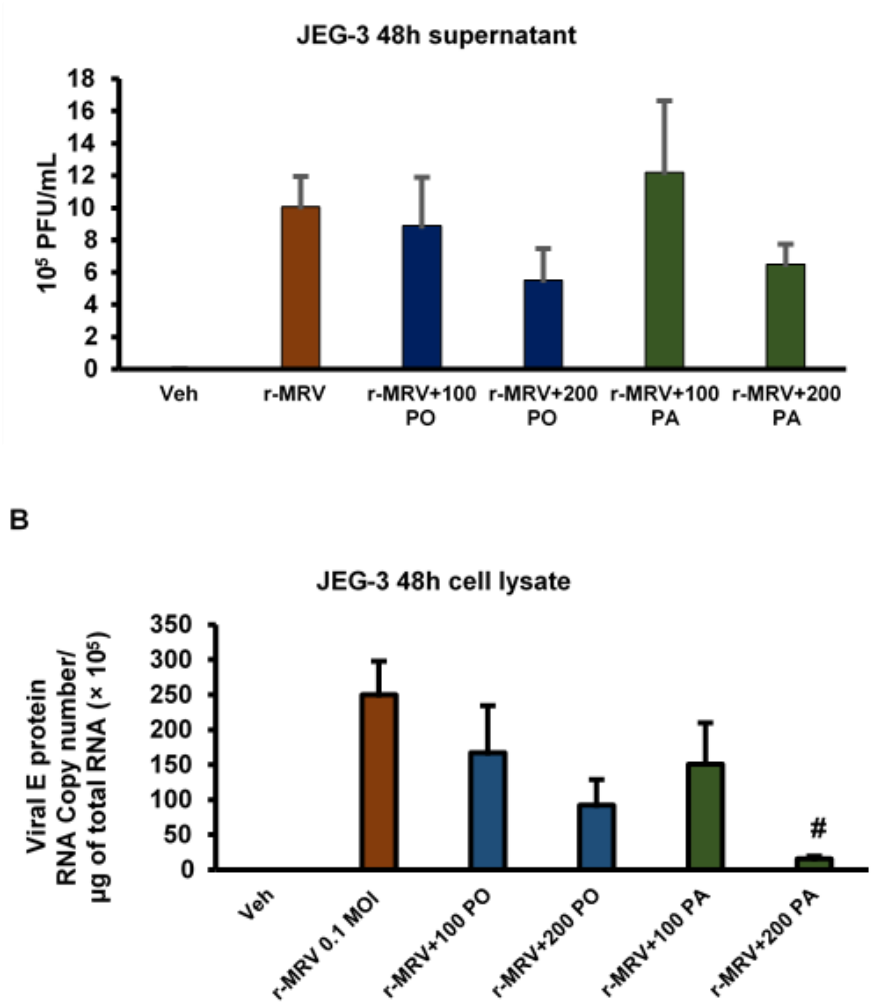
Fig.3.3. Treatment of the placental cells with palmitoleate after ZIKV infection interferes with viral replication.

Presence of viral envelope RNA copy number in the cell culture supernatant of both (A) JAR and (B) HTR-8 cells infected with 0.1 and 1.0 MOI of ZIKV MR766 strain 72h and 96 hpi respectively. When MRV infected cells were treated with palmitoleate (100-200µM), there was significant reduction in the viral envelope RNA copy number in the cell culture supernatant with both 0.1 and 1 MOI in JAR cells and with 1 MOI in HTR-8 cells (A-B). Presence of viral envelope RNA copy number both in JAR (C) and HTR-8 (D) cell lysate infected with 0.1 and 1.0 MOI of ZIKV MR766 strain 72 and 96 hpi respectively. When the infected cells were treated with 100-200µM of palmitoleate, there was a trend showing reduced viral envelope RNA per microgram of total RNA in both JAR cell lysate (C) and HTR-8 cell lysates (D). Data represents mean ±SEM, n=3. #P<0.05 compared to ZIKV infected cells. (E-G) Viral E protein staining in JEG cells infected with 0.1 MOI r-MRV and in cells treated with 100 or 200µM palmitoleate, 48hpi. (E) ZIKV infected cells show green fluorescence indicating presence of viral E protein. (F) ZIKV infected JEG-3 cells treated with 200µM palmitoleate and show a reduction in the intensity of viral E protein staining. (G) Infected JEG-3 cells treated with 100-200µM palmitate showed increased intensity of viral E protein staining. Nuclei were stained with DAPI. (H) Immunoblot analysis showed dramatic increase in viral E protein expression with ZIKV infection in JEG-3 cells compared to vehicle uninfected cells. Treatment of 200µM palmitoleate to ZIKV infected cells showed reduced viral E protein expression compared to the ZIKV infected cells alone.

Immunofluorescence analysis of Zika Viral E protein: We, next tested the expression of viral E protein in JEG-3 cells 48 hpi with 0.1MOI of r-MRV. There was an increase in the expression of Zika viral E protein in JEG-3 cells with 0.1 MOI r-MRV infection

compared to the uninfected vehicle cells (Fig.3.3E). The viral E protein expression was dramatically reduced in JEG-3 cells treated with palmitoleate (200µM) 48 hpi compared to ZIKV infected cells alone. The viral E protein expression was dramatically increased in JEG-3 cells treated with palmitoleate (100µM) 48 hpi and palmitate (100-200µM) (Fig.3.3F). The number cells in the 200µM palmitate treated cells was significantly less which was comparable to infected cells alone (Fig.3.3G). Western blot analysis of JEG-3 cells infected with 0.1 MOI r-MRV showed dramatic increase in viral E protein and treatment of 100- 200 µM palmitoleate showed a decrease in viral E protein expression. Treatment of palmitate (100-200µM) did affect the viral E protein expression 48 hpi (Fig.3.3H). Similarly, there was non-significant decrease in pfu/mL in cell culture supernatant from 200µM palmitoleate treated cells for 48hpi in JEG-3 cells. Palmitate treatment at 100 or 200 µM concentration altered JEG3 viral load in cell cuture supernatant (Supplementary figure 2A). Palmitoleate treatment (100-200µM) in JEG-3 cells after infection with 0.1 MOI r-MRV showed a non-significant decrease in viral (E gene) RNA copy number in cell lysate. We also observed a non-significant decrease in viral E gene RNA copy number in the cell lysate with 100µM palmitate treatment. However, JEG-3 cell lysate after 200µM palmitate treatment to 0.1 MOI r-MRV infected cells showed a significant reduction in the viral RNA copy number compared to ZIKV infected cells alone (Supplementary Fig.3.2B).

Supplementary Figure 2



Supplementary Fig.2. (A) Plaque assay using cell culture supernatant showed a trend in decreased pfu/ml in 200 µM palmitoleate treated +ZIKV infected cells. Treatment of 100 µM palmitate for 48h with 0.1 MOI r-MRV infected cells showed a trend towards increase in pfu/ml but in contrast 200µM palmitate treatment showed a trend towards reduction in pfu/ml. Data represents mean ±SEM, n=3. #P<0.05 compared ZIKV infected cells. (B) Similarly, viral envelope copy number in cell lysate with 200 µM palmitoleate treatment showed a trend towards reduction when compared 0.1 MOI r-MRV infected cells alone but 200µM palmitate treated cells showed a significant reduction in the viral envelope RNA copy number 48hpi. Data represents mean ±SEM, n=3. #P<0.05 compared to ZIKV infected cells.

Palmitate does not protect against ZIKV-induced ER stress and apoptosis:

Treatment of JEG-3 cells with palmitate, a saturated fatty acid, after ZIKV infection either with r-MR or PR strains did not protect against ZIKV-induced trophoblast apoptosis (Fig.3.4). There was significant reduction in the percent apoptotic nuclei and caspase 3/7 activation with palmitoleate treatment and this protection was not observed with the treatment of palmitate. (Fig.3.4A-C). Additionally, treatment of palmitate to r-MRV infected JEG-3 cells caused significant increase in percent apoptotic nuclei levels compared to r-MRV infection alone (Fig.3.4A). These results suggest a unique protective property of palmitoleate against ZIKV-induced trophoblast apoptosis and that are not observed with the treatment of palmitate.

Figure 4

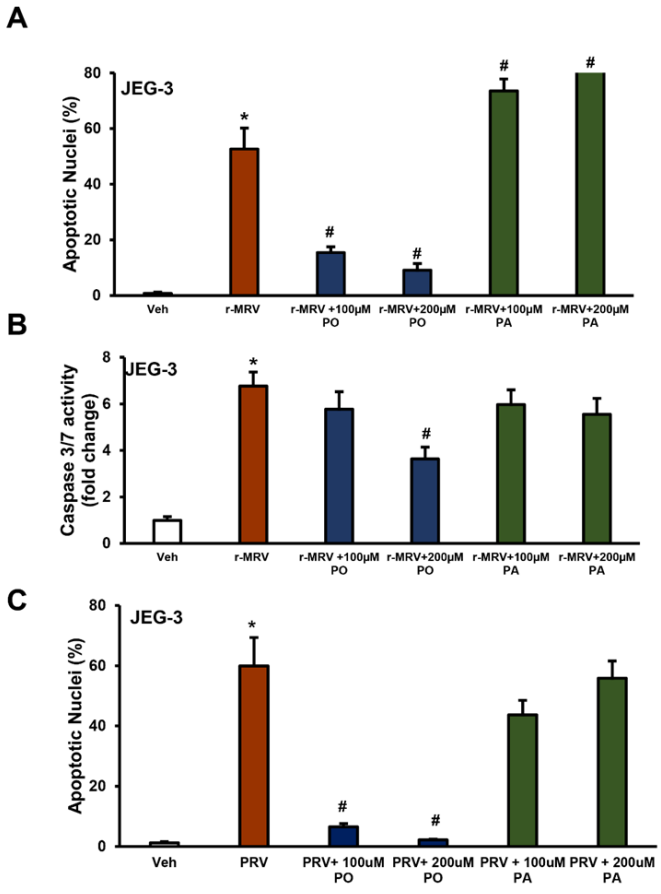


Fig.3.4. Palmitoleate protects against ZIKV-induced apoptosis but not palmitate.

(A-B) There was significant reduction in (A) percentage apoptotic nuclei and (B) caspase 3/7 activation in JEG-3 cells treated with palmitoleate 48hpi with 0.1 r-MRV suggesting the protective role of palmitoleate against ZIKV-induced apoptosis. When the JEG-3 cells were treated with palmitic acid 48hpi, there was significant increase in the percent apoptotic nuclei and a trend towards increase in caspase 3/7 activation suggesting that palmitate did not protect against ZIKV-induced apoptosis. Data represents mean ±SEM, n=3 for percentage apoptotic nuclei and n=4 for caspase 3/7 activity.*P<0.05 compared to uninfected vehicles cells, #P<0.05 compared ZIKV infected cells. (C) Similarly, in JEG-3 cells with 1.0 MOI of PRV for 48h the percent apoptotic nuclei was significantly reduced in 100-200 µM palmitoleate treated PRV infected cells compared to ZIKV infected cells alone, however treatment of palmitate to ZIKV infected cells did not prevent ZIKV-induced apoptosis. Data represents mean ±SEM, n=3.*P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.

Palmitoleate improves cell viability in ZIKV infected trophblasts: The percent cell survival measured using crystal violet shows a significant reduction in the percent cell survival with 0.1 MOI r-MRV infection in JEG-3 cells, 48 hpi when compared to uninfected vehicle cells. The percent cell survival was significantly increased with the treatment of 100 or 200µM palmitoleate post infection and this protection was not observed with the treatment of palmitate after ZIKV infection (Fig.3.5A). Similarly, the percent cell survivability assessed using MTT also showed significant reduction in cell survival with 0.1 MOI r-MRV in JEG-3 cells when compared to uninfected vehicle cells. Treatment of palmitoleate to JEG-3 cells infected with 0.1 MOI r-MRV showed a significant increase in cell survivability with 200 µM concentration wheras as 100 µM concentration showed a non-significant increase in percent cell survival. Surprisingly, palmitate treatment in infected cells at 200µM concentration showed a slight but significant increase in percent survival (Fig.3.5B).

Figure 5

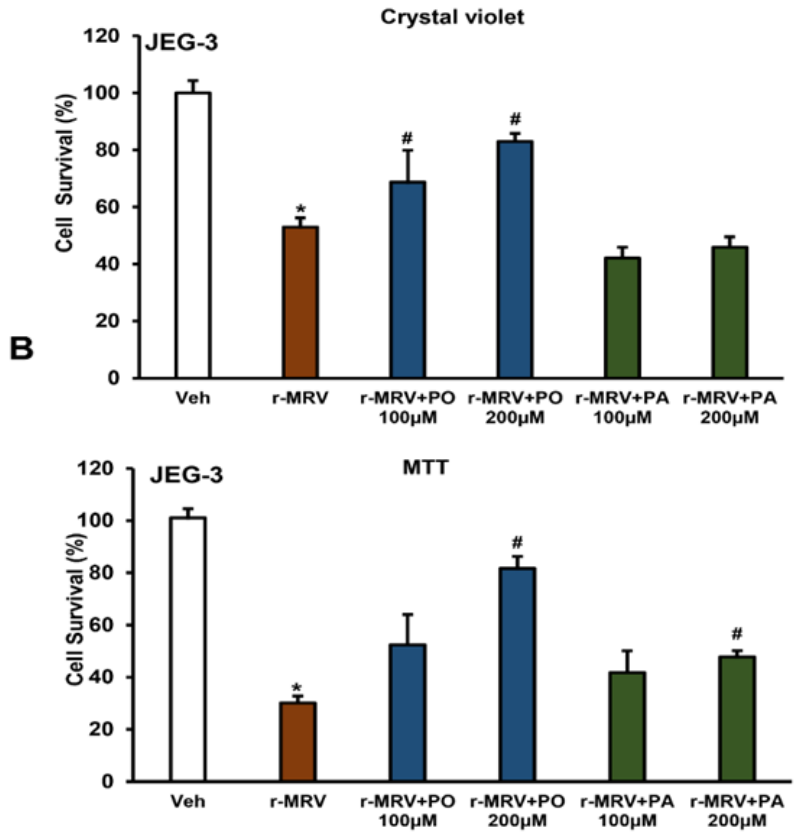


Fig.3.5. Palmitoleate improves cell viability in ZIKV infected cells.

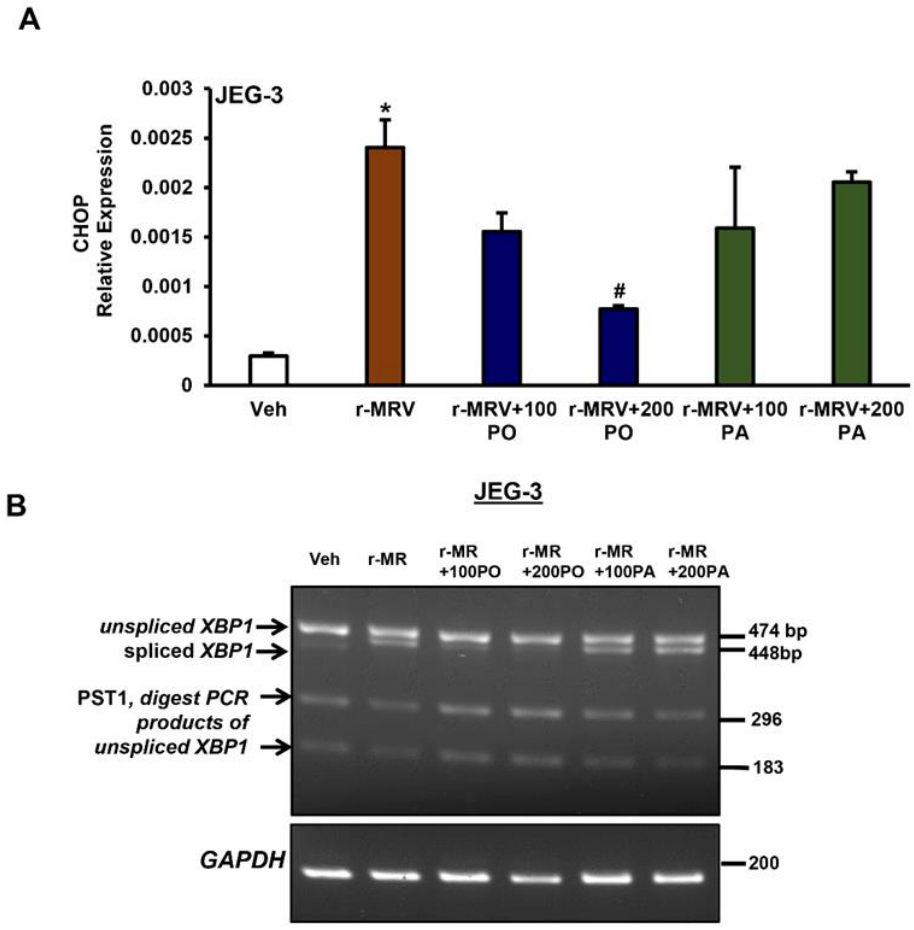
(A) The percent survival using crystal violet assay was significantly reduced with 0.1MOI r-MRV when compared to uninfected vehicle cells. The percent cell survival was significantly increased with 100 or 200µM of palmitoleate treatment, but not seen with100-200µM palmitate treatment 48hpi. (B) The percent cell survivability using MTT showed significant improvement in cell survivability with 200 µM palmitoleate treatment in ZIKV-infected cells. Whereas 200µM palmitate treatment in ZIKV infected cells also showed a significant increase in cell survivability. Data represents mean ±SEM, n=3 for percent cell viability using crystal violet assay and n=4 for MTT assay. *P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.

Palmitoleate protects against ZIKV-induced endoplasmic reticulum (ER) stress: We have earlier demonstrated that ZIKV infection of trophoblasts induces an increase in the

levels of C/EBP homologous protein (CHOP) mRNA and Spliced X box associated protein-1 (XBP1) mRNA, which are key markers of ER stress (30). We assessed the activation of ER stress markers namely CHOP mRNA expression and *XBP1* gene splicing with palmitoleate or palmitate treatment to ZIKV-infected JEG-3 cells. We observed a significantly higher levels of CHOP mRNA expression with 0.1 MOI r-MRV infection in JEG-3 cells, 48 hpi compared to uninfected vehicle cells. Treatment of palmitoleate at 200 µM final concentration to the ZIKV-infected cells showed a significant reduction in the expression of CHOP. However, treatment of 100 µM palmitoleate showed only a trend towards decrease in CHOP mRNA expression compared to ZIKV-infected cells (Fig.3.6A). Whereas, treatment of 100 or 200 µM palmitate to ZIKV infected cells did not significantly decrease the expression of CHOP (Fig.3.6A). We next investigated *XBP1* mRNA splicing levels (~448 bp band) which is an additional indicator of ER stress in cells. Spliced *XBP1* mRNA levels were elevated with 0.1 MOI r-MRV infection in JEG-3 cells compared to uninfected vehicle cells. Palmitoleate treatment (100 or 200 µM) was able to reduce the extensive *XBP1* mRNA splicing seen in the ZIKV infected cells. However, treatment of palmitate did not prevent the increased levels of spliced XBP1 caused due to ZIKV infection (Fig.3.6B). The relative band intensity of spliced XBP1 showed a significant increase in percent ratio of spliced XBP1/GAPDH in ZIKV infected cells when compared to uninfected vehicle cells. There was a trend towards increase in spliced XBP1/GAPDH in palmitate treated cells compared to ZIKV infection alone. However, supplementation of palmitoelate signicantly decreased percent ratio of spliced XBP1/GAPDH (Fig.3.6C). A trend towards increase was observed with the percent ratio of unspliced XBP1/GAPDH in

palmitoleate treated ZIKV infected cells when compared to ZIKV infected cells alone or ZIKV infected cells treated with palmitate (Fig.3.6D). Thus, treatment of palmitoleate appears to be a protective nutrient therapy against ZIKV-induced ER stress and apoptosis in trophoblasts.

Figure 6



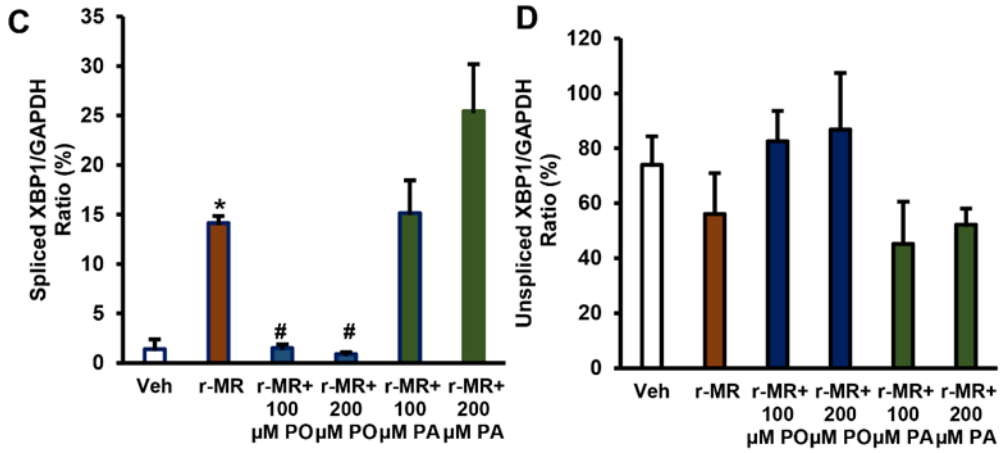


Fig.3.6. Palmitoleate protects against ZIKV-induced ER stress but not palmitate.

(A) CHOP mRNA expression was significantly upregulated with 0.1 MOI r-MRV 48hpi in JEG-3 cells compared to uninfected control cells. There was significant reduction in CHOP mRNA expression in JEG-3 cells treated with 200µM palmitoleate 48hpi with ZIKV suggesting protective role of palmitoleate against ZIKV-induced apoptosis and a trend towards decreased expression with 100µM palmitoleate treatment but this was not seen with the treatment of 100 or 200µM palmitate to r-MRV infected cells. (B) Similarly, there was increase in XBP1 mRNA splicing in infected cells (band ~448bp) when compared to uninfected vehicle cells. There was significant inhibition of XBP1 mRNA splicing with the treatment of 100 or 200µM of palmitoleate in ZIKV infected cells. However the protective properties was not seen with the treatment of palmitate to ZIKV infected cells. (C) Quantified levels of spliced XBP1 mRNA (448 bp) relative to GAPDH (Spliced XPB1/GAPDH). (D) Quantified levels of unspliced XBP1 mRNA (474bp) relative to GAPDH (Unspliced XPB1/GAPDH). Data represents mean ±SEM, n=3. *P<0.05 compared to uninfected vehicles cells, #P<0.05 compared to ZIKV infected cells.

3.5 Discussion

Zika virus is known to cause apoptosis via sustained ER stress in the trophoblasts (30).

The principal findings of the present study are 1) palmitoleate, an omega 7 monounsaturated fatty acid significantly reduces ZIKV infection-induced trophoblast apoptosis; 2) treatment of palmitoleate interferes with ZIKV replication in trophoblasts; 3) palmitoleate treatment after ZIKV infection in trophoblasts downregulates the

activation of ER stress markers that occurs due to viral protein overload, and 4) palmitate, a saturated fatty acid with similar carbon structure to palmitoleate augments cell death in ZIKV-infected trophoblasts. The schematic representation of palmitoleate protection against ZIKV-induced trophoblast apoptosis is shown in Figure 3.7.

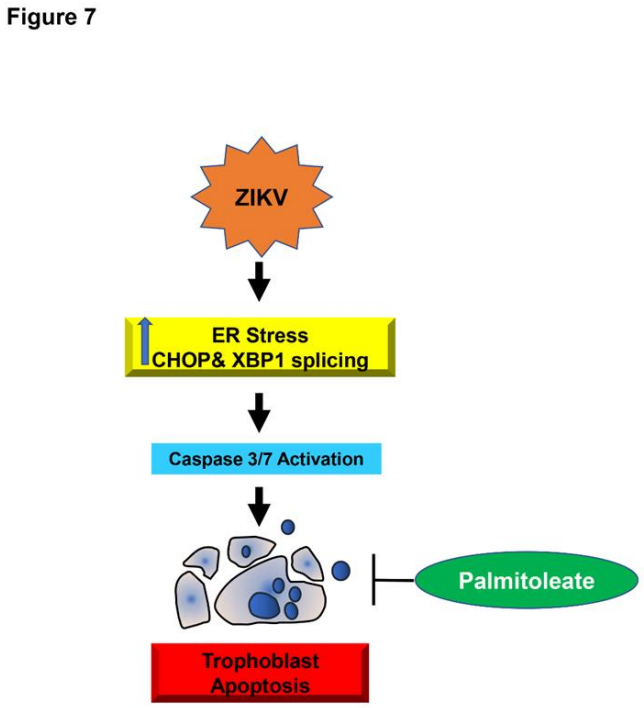


Fig.3.7. The schematic diagram represents palmitoleate protection against ZIKV-induced ER stress and apoptosis in placental trophoblasts.

ZIKV infection in trophoblasts elicits ER stress via the upregulation of CHOP and XBP1 mRNA splicing which in turn activates apoptosis. Supplementation of palmitoleate protects against ZIKV-induced ER stress and trophoblast apoptosis.

ZIKV infection from mother to the developing fetus is detrimental in causing Congenital Zika Syndrome (4). Trophoblasts, the epithelial cells of the placenta express receptors such as AXL, Tyro3, and TIM1 that facilitate the entry of ZIKV into these cells (33,34). ZIKV infection of *Infar1* knock out mice shows that ZIKV is able to breach the placental barrier and affects the survivability of the fetuses (12). Similarly, a human STAT2 knock-

in, immunocompetent mouse model shows that a mouse adapted ZIKV strain belonging to African lineage was able to cross the placental barrier and blood brain barrier (35). Therefore, transplacental route of transmission from mother to the fetus plays a crucial role in the disease process (36,37). In our previous study, we have shown that ZIKV induces a caspase-dependent trophoblast apoptosis as evidenced by significant increase in percent apoptotic nuclei and caspase 3/7 activation following ZIKV infection. Further, inhibition of caspases activity using Z-VAD-fmk prevented ZIKV-induced placental trophoblast apoptosis (30). ZIKV is also known to cause changes in the sphingolipid metabolism in the host cells. Ceramide, a sphingolipid is already known to be associated with apoptosis and have been found to be essential for ZIKV replication cycle in the host cells (38,39). Ceramides are also known cause ER stress and affect overall lipid metabolism in hepatocytes (40). However, protective role of palmitoleate supplementation against ZIKV-induced ER stress and apoptosis via alteration of sphingolipid metabolism needs further investigation.

There are several potential vaccine candidates, therapeutic drugs and nutraceutical compounds under investigation for protection and treatment against ZIKV infection (41-45). Since the prospective target population who needs protection during outbreaks involves pregnant women, this poses challenges regarding the safety of the vaccine candidates or the drugs that can ensure safety to both the mother and the developing baby without any adverse reactions (46-50). In contrast, nutrient compounds can be an alternative strategy to combat viral infections in the context of safety during pregnancy. For example, 25-hydroxy cholesterol, an oxysterol metabolite, which plays a critical role in cholesterol biosynthesis and innate immune response was shown to be protective

against ZIKV-induced microcephaly in type I interferon α/β receptor knockout (*Infar*$^{-/-}$) mice (51). In an another study, it was observed that natural polyphenols like delphinidin and epigallocatechin gallate have anti-viral properties against flavivirus including ZIKV (52). Curcumin, a polyphenol present in turmeric tubers was also found to inhibit the attachment of ZIKV to the host cells (53). Naringenin, a flavonoid compound seen in citrus fruits had anti-viral properties against ZIKV infection by interacting with the protease domain of the virus (54). In the present study, we have established the protective role of palmitoleate against ZIKV-induced apoptosis in placental trophoblasts in an *in vitro* model.

Palmitoleate (16:1 n-7) is rich in dietary sources such as sea buckthorn oil and macadamia nuts (55). In mammals, palmitoleate can be synthesized by stearoyl-CoA desaturase 1 (SCD1) enzyme from the saturated fatty acid, palmitate (56). Palmitoleate is abundant in adipose tissue, blood cells and it is a part of cell membrane structure (57). Palmitoleate plays an important role in maintaining metabolic health and homeostasis by acting as a lipokine (58). Palmitoleate decreases fat deposition in liver and enhances insulin sensitivity (57). Studies have shown that palmitoleate protects against free fatty acid-induced hepatocyte lipoapoptosis (23) and trophoblast lipoapoptosis (59), respectively. Supplementation of palmitoleate has shown to be effective against non-alcoholic fatty liver disease and atherosclerosis in mouse models (60,61). Palmitoleate can skew pro-inflammatory state to anti-inflammatory state of macrophages in mice fed with high fat diet via AMP activated protein kinase signaling (62). Previous studies also suggest that monounsaturated fatty acids such as palmitoleate and oleate inhibit replication of enveloped bacteriophage phi6 and PR4 (63,64). Further, palmitoleate has

also been shown to protect against tunicamycin- and palmitate-induced ER stress and apoptosis in hepatocytes and pancreatic beta cells, respectively (23,24). Our data in the present study supports the protective role of palmitoleate against ZIKV-induced ER stress and apoptosis in placental trophoblasts and therefore could likely serve as a therapeutic candidate or preventive nutrient compound for ZIKV infection in pregnant mothers in disease infection prone areas.

Our data shows a proof that palmitoleate prevents ZIKV-induced trophoblast apoptosis. We also saw reduction in viral RNA copy number in the cell culture supernatant from JAR (0.1 and 1MOI) and HTR-8 (1 MOI) infected cells following palmitoleate treatment. On the other hand we saw a trend towards reduction viral RNA copy number in both JAR and HTR-8 cell lysate, this could be due to the fact that viral particles were already released from the cells to the culture supernatant. We also saw reduction in the expression of viral E protein in JEG-3 cells infected with 0.1 MOI r-MRV and with a co-treatment of 200µM palmitoleate. Similarly, 200µM palmitoleate treatment showed improved cell survivability and reduced viral E protein staining, further suggests that it could interfere with the viral replication. Viral E protein in ZIKV also has a lipid component and might also possibly be altered with palmitoleate treatment, which require further investigations.

Although we saw significant reduction in viral E gene copy number in 200µM palmitate treated JEG-3 cell lysate, this might be due to the fact that there is less viable cells for viral replication in palmitate treated cells as observed in apoptotic nuclei and cell viability assay. The MTT assay measures active mitochondrial dehydrogenase and we showed that mitochondrial enzyme activity is compromised in ZIKV infected cells alone

whereas palmitate treatment to ZIKV infected cells resulted in intact mitochondrial enzyme activity. Further, to substantiate this phenomenon a recent study suggests that low levels of palmitate supplementation can increase mitochondrial function (65). Also the use of crystal violet for cell viability assessment has been shown to be more reliable than MTT assay (66).

ZIKV is also known to alter the lipid homeostasis of the placenta by altering the organelles in the cells by forming virus replicating complexes enclosed in vesicles. ZIKV also resulted increase in accumulation of large lipid droplets both in infected and uninfected bystander placental cells (15). However further studies are required to elucidate the mechanism behind the protective role of palmitoleate by affecting the viral replication. This could be possible through ways such as i) hindrance against Zika viral entry receptor binding; ii) E protein lipid component; or iii) inhibition of specialized viral replication complex are warranted.

Studies have shown that palmitate, a saturated fatty acid can either promote viral infection (67) or have anti-viral properties (68) via autophagy flux mechanism. We found that palmitate treatment augmented apoptosis in ZIKV infected trophoblasts. A study using Influenza A virus model showed that palmitate supplementation to the cells can enhance viral replication (69). Similarly, Rift Valley Fever virus-infected cells were shown to activate AMP-activated protein kinase (AMPK) and decreases viral replication by limiting fatty acid synthesis, treatment of palmitate helped in initiating fatty acid synthesis and aids in replication of the Rift Valley Fever virus (70). In our study, palmitate treatment to ZIKV infected cells reduces the cell viability and probably inducing an alternate cell death pathway in addition to apoptosis.

Palmitoleate is known to activate cell survival pathways and has been shown to protect against metabolic syndrome (57). Palmitoleate plays a protective role by modulating peroxisome proliferator-activated receptor alpha (PPARα), an important transcription factor that regulates fatty acid oxidation by activating AMPK which in turn improves glucose metabolism in liver cells of mice fed with high fat (56,71). Further, post-translational modification of Wnt protein with palmitoylation results in a signaling pathway that activates β-catenin, a cell survival signal (72,73). Several studies have also shown that palmitoleate supplementation helps in improving metabolic diseases in humans such as cardiovascular diseases and diabetes mellitus (74,75). Our results study suggests that palmitoleate treatment in ZIKV infected trophoblasts is protective against ER stress and apoptosis, thereby considerably improves cell survival. However further mechanistic studies are underway in elucidating the protective role of palmitoleate against ZIKV-induced trophoblast apoptosis.

3.6 Conclusion

Palmitoleate is protective against ZIKV-induced ER stress and apoptosis in trophoblasts. The mechanism of palmitoleate protection against ZIKV-induced ER stress and apoptosis is either via direct interference of viral replication or by the activation of cellular survival pathways or a combination of both, requires which needs further investigations.

3.7 References

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CHAPTER 4: PROTECTIVE ROLE OF PALMITOLEATE AGAINST ZIKA VIRUS-INDUCED INFLAMMATION IN PLACENTAL TROPHOBLASTS

4.1 Abstract

Zika virus (ZIKV) is known to cause an inflammatory response in the brain, retina and placenta. The production of excessive inflammatory cytokines can be detrimental to fetal development. ZIKV infection also triggers inflammasome activation to exploit inflammatory pathways, thereby evades immune responses by the host. In this study, we explored the protective role of palmitoleate against inflammatory response in ZIKV infected trophoblasts. The pro-inflammatory cytokine IL-1β mRNA expression was significantly downregulated in 200μM palmitoleate-treated trophoblasts after ZIKV infection. We also found that lipid droplet accumulation was higher in ZIKV infected cells with palmitoleate treatment when compared to palmitate treatment in ZIKV infected cells or ZIKV infection alone. Further studies are required to elucidate the protective properties of palmitoleate against ZIKV-induced inflammatory response in trophoblasts.

4.2 Introduction

ZIKV is known to induce an inflammatory response in the nervous system, the eye, the placenta, and the male and female reproductive organs (1-5). ZIKV infection in the human placenta causes extensive alterations in lipid metabolism to favor its replication along with the inflammatory response (3). ZIKV infected adult populations can develop neurological problems associated with inflammation including Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy, acute transient polyneuritis, meningoencephalitis, myelitis, acute disseminated encephalomyelitis and encephalopathy

(6). ZIKV induced inflammatory response is mainly attributed to IL-1β secretion associated with NLRP-3 inflammasome activation (7). ZIKV uses NLRP-3 inflammatory pathway via NS-1 to thwart the host's immune response against it (8). IL-22 was found to be a key cytokine that impedes cell-mediated immune responses against ZIKV and aggravates inflammation of the brain in newborn mice (9). Another study in neonatal mice suggests that ZIKV persists in the central nervous system even after a year resulting in chronic inflammation (10).

It is known that lipid droplet formation helps to prevent activation of inflammatory cell signaling pathways while lipolysis favors inflammatory response (11). It is also known that palmitoleate can modulate lipid metabolism by enhancing lipogenesis and fatty acid oxidation (12). Further, lipid droplet formation is known have a protective role against viral infection (13). In this study, we hypothesize that supplementation of palmitoleate in ZIKV infected cells would increase lipid droplet accumulation and protect against ZIKV-induced inflammatory cytokine response.

4.3 Materials and methods

Materials:

Adipose differentiation-related protein **(**ADRP) antibody (# 10R-A117ax) procured from Fitzgerald, MA. ZIKV E protein antibody was obtained from Gene Tex (# GTX133314), CA. Alexa flour 488 conjugated anti-rabbit antibody (# A11008) and Alexa flour 647 conjugated anti-mouse antibody (# A21237) were obtained from Invitrogen, CA. Oil red

O (#O0625) and saponin (#47036) were from Millipore Sigma, MO. Paraformaldehyde 16% (#15710) procured from Electron Microscopy Sciences, PA.

Quantitative Real time PCR: JEG-3 cells infected with 0.1 MOI r-MRV and treated with palmitoleate for 48h. TriZOL reagent (Thermo Scientific) was used to isolate RNA from the scrapped cells and subjected cDNA synthesis with superscript II reverse transcriptase (Invitrogen). Target IL-1β mRNA expression was amplified by primer and normalized with 18S rRNA expression using SYBR green assay as described by (14).

Immunofluorescence analysis: Collagen-coated coverslips kept inside 24 well plates were used for JEG-3 cell attachment and treatment of the cells with fatty acids after 1 h of virus adsorption. After 48 h of incubation, cells were washed with PBS and fixed with 3% paraformaldehyde solution containing 100mM piperazine-N, N′-bis(2-ethanesulfonic acid) (PIPES), 3mM $MgSO_4$ and 1mM ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) for 30mins. After fixation cells were washed with PBS and permeabilization was done with 0.5% saponin (Millipore Sigma) in PBS for 10mins. Cells were once again washed with PBS and blocked using PBS containing 5% glycerol, 5% FBS and 0.01% sodium azide for 30mins. Overnight incubation at 4^oC with anti-ADRP antibody (1:10) and antibody raised against ZIKV E protein (1:100) was done. Excess unbound antibody was washed away using PBS wash, 3 times. Anti-mouse secondary ab tagged with Alexaflour 647 (1:100) and anti-rabbit secondary ab tagged with Alexaflour 488 (1:500) were used for 2 h at room temperature. The cells were washed with PBS counterstained with nuclear DAPI stain for 5mins followed by a final

wash with PBS. The coverslips were carefully removed, blotted and mounted onto glass slides and visualized under Nikon A1R-Ti2 confocal system.

Oil Red O staining: JEG-3 cells infected with 0.1r-MRV and treated with either palmitoleate or palmitate in 24 well plates were fixed with 10% formalin for 10mins followed by PBS wash. Staining with a working solution of oil red O for 15mins (4ml distilled water+6ml stock oil red O solution). The stock solution was prepared by dissolving 0.5g of oil red O in 100 ml of 99% isopropanol. After staining, the plates were washed with distilled water three times and mounted with coverslips. Images were acquired using EVOS FL Auto Cell Imaging System.

Statistical analysis: Analysis of variance (ANOVA) with post-hoc Bonferroni corrections was employed for comparisons of multiple groups. P value <0.05 was considered statistically significant.

4.4 Results

Palmitoleate treatment (200μM) in ZIKV infected JEG-3 cells results in attenuation of pro-inflammatory cytokine IL-1β: JEG-3 infected with 0.1 MOI r-MRV treated either with palmitoleate or palmitate treatment for 48 hpi were used to measure mRNA levels of IL-1β. We saw that IL-1β mRNA expression levels in 200μM palmitoleate treated cells after ZIKV infection was significantly downregulated when compared to ZIKV infected cells treated with 100μM palmitoleate and ZIKV infected cells alone (Fig.4.1). This result suggests that 200μM palmitoleate treatment in ZIKV infected JEG-

3 cells attenuates pro-inflammatory cytokine IL-1β expression in response to viral infection.

Figure 1

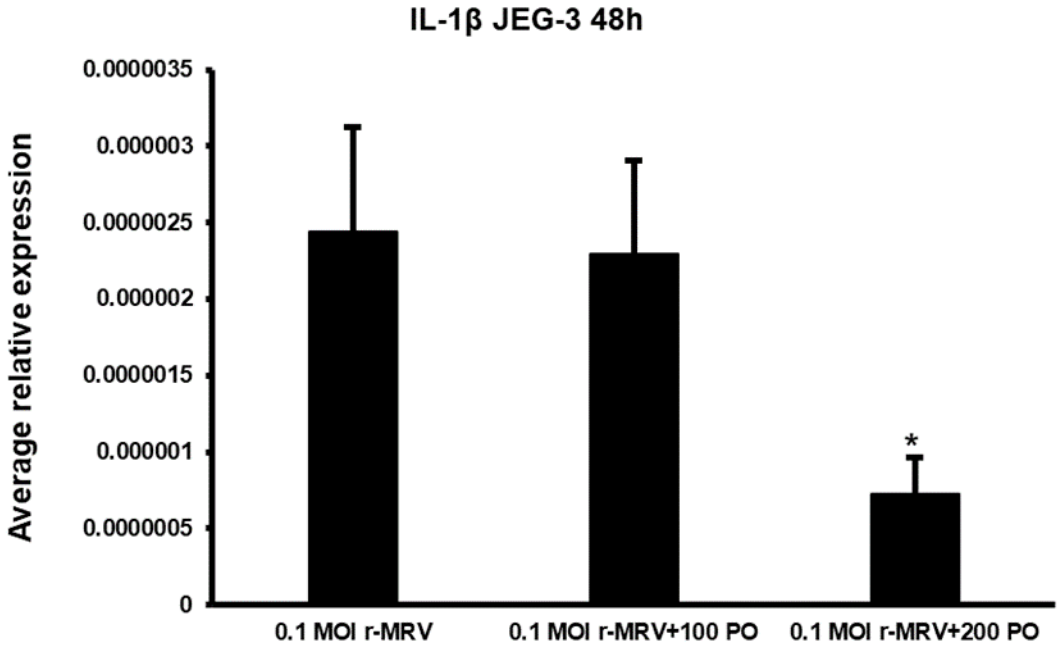


Fig.4. 1.Palmitoleate (200μM) treatment in ZIKV infected JEG-3 cells results in attenuation of pro-inflammatory cytokine IL-1β.

JEG-3 cells infected with 0.1 MOI r-MRV were treated with 100-200μM palmitoleate for 48h. ZIKV infected JEG-3 cells with 200μM palmitoleate supplementation showed a significant reduction in the IL-1β mRNA expression when compared to infected cells alone and ZIKV infected cells treated with 100μM palmitoleate. Uninfected vehicle cells showed a ct value of N/A or less than 32. Data represent ± SEM, n=3, * $P<0.05$ compared to ZIKV infected cells, statistical comparison by ANOVA with post hoc Bonferroni correction.

Palmitoleate increases lipid droplet accumulation and decreases viral E protein expression in JEG-3 cells: JEG-3 cells were infected with r-MRV (0.1 MOI) and treated

with either palmitoleate or palmitic acid. After 48 h of infection expression of both viral E protein and ADRP (biomarker for lipid droplets) were analyzed by immunofluorescence. Uninfected vehicle cells showed no ZIKV E protein expression along with little ADRP staining (Fig.4.2A). ZIKV infected cells showed intense viral E protein staining whereas little ADRP staining compared to vehicle cells (Fig.4.2B). JEG-3 cells treated with 100µM palmitoleate 48hpi showed both viral E protein sating as well as ADRP staining (Fig.4.2C). Interestingly, ADRP staining was seen in healthy cells without viral E protein expression. While JEG-3 cells treated with 200µM palmitoleate 48hpi showed high levels of ADRP expression and very low levels of viral E protein expression compared to ZIKV infected cells alone (Fig.4.2D). JEG-3 cells treated with 100µM palmitate 48hpi showed intense viral E protein staining and low ADRP staining (Fig.4.2E). ZIKV infected cells when co-treated with 200µM palmitate showed lesser viral E protein staining intensity when compared to 100 µM palmitate along with low levels of ADRP expression (Fig.4.2E-F).

Figure 2

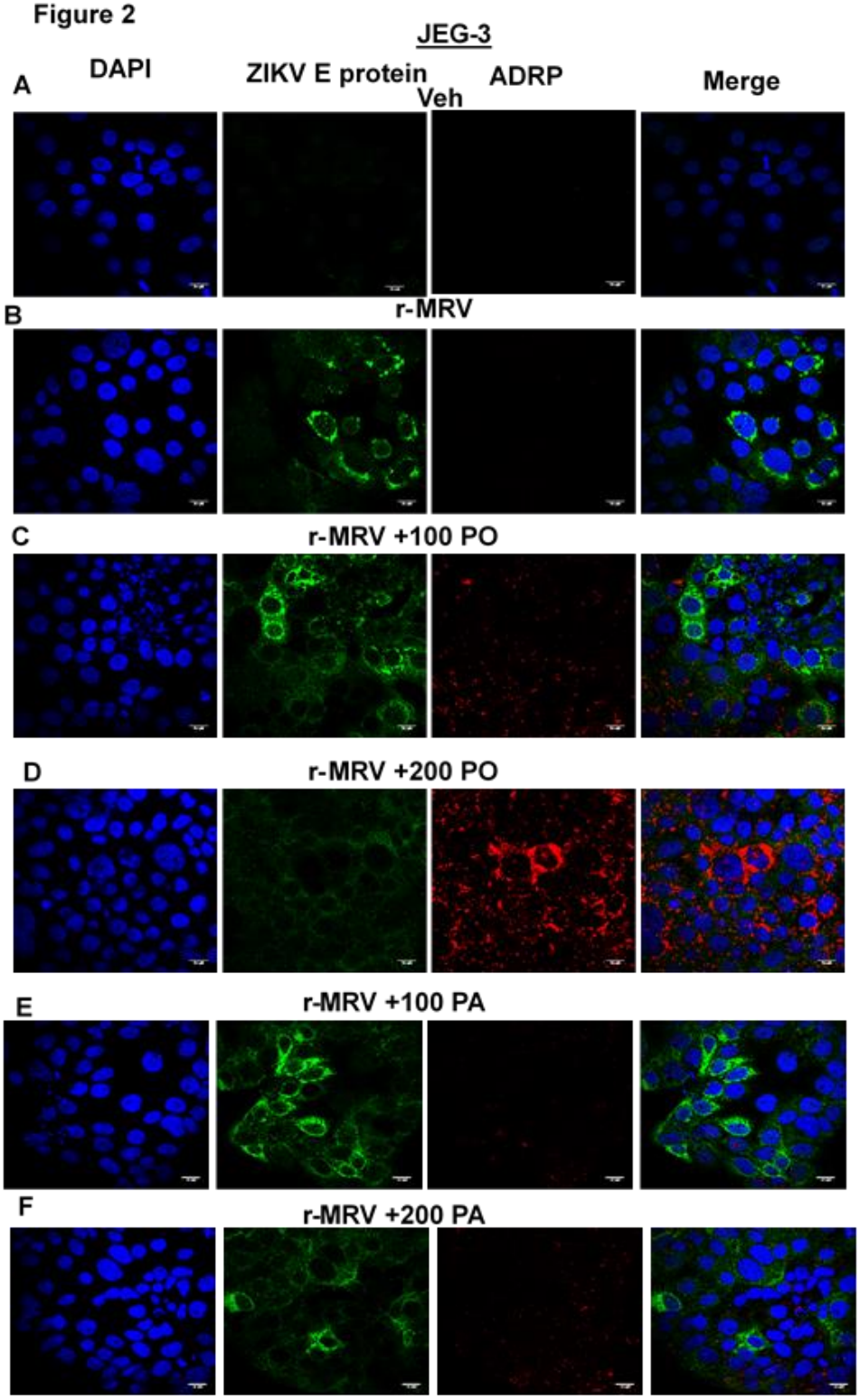

JEG-3
DAPI
ZIKV E protein
ADRP
Merge
A
Veh
B
r-MRV
C
r-MRV +100 PO
D
r-MRV +200 PO
E
r-MRV +100 PA
F
r-MRV +200 PA

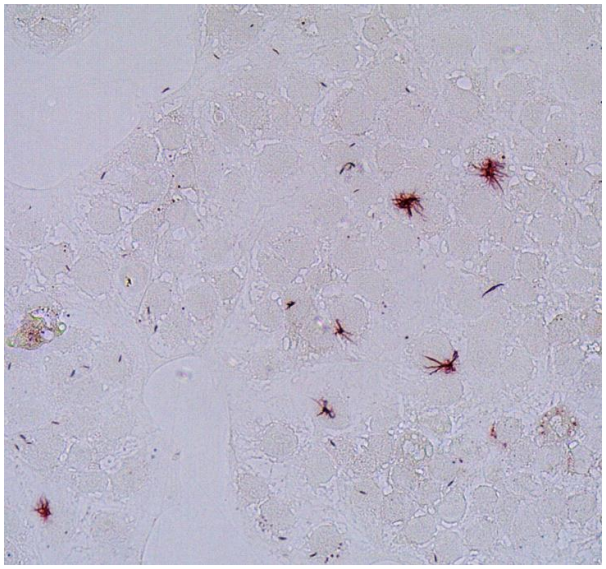
Fig.4. 2.Palmitoleate increases lipid droplet accumulation and decreases viral E protein expression in JEG-3 cells.

Representative images from immunofluorescence analysis with DAPI (blue), ZIKV E protein (green), ADRP (red) and merge panels respectively (A) Vehicle: Uninfected JEG-3 cells showed neither viral E protein expression nor lipid droplet accumulation. (B) r-MRV: JEG-3 cells infected with 0.1 r-MRV for 48h showed ZIKV E protein expression. (C) r-MRV+100 PO: JEG-3 treated with 100µM palmitoleate for 48hpi showed cells with ZIKV E protein expression and cells without viral E protein expression showed lipid droplet accumulation. (D) r-MRV+200 PO: JEG-3 treated with 200µM palmitoleate for 48hpi showed dramatic accumulation of lipid droplets along with very low levels of viral E protein expression. (E-F) r-MRV+100 PA, r-MRV+200 PA: JEG-3 cells treated with 100-200µM palmitate for 48hpi showed viral E protein expression coupled with very low levels of lipid droplet formation.

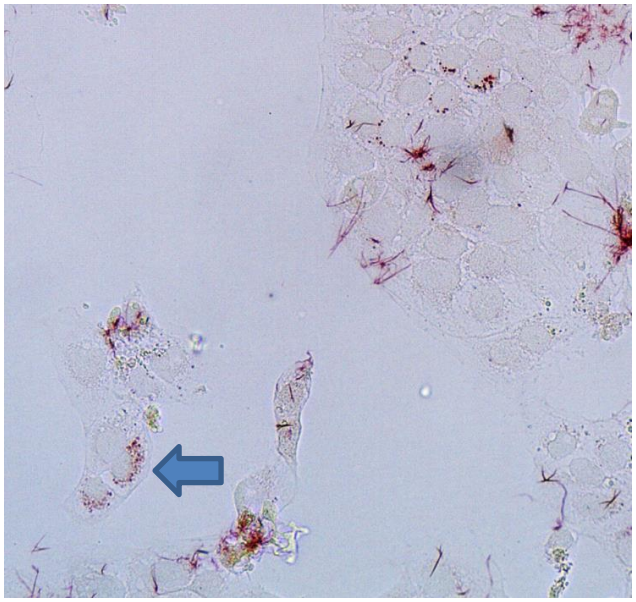
Lipid droplet staining in ZIKV infected JEG-3 cells using oil red O staining: Lipid droplets were stained with oil red O in r-MRV (0.1 MOI) infected JEG-3 cells co-treated with either palmitoleate or palmitate for 48 h. Very low levels of lipid droplets were noticed in uninfected vehicle cells (Fig.4.3A) whereas ZIKV infected cells showed lipid droplets concentrated in the healthy cells surrounding areas that showed cytopathic effect as shown in Fig.4.3B. Both 100 and 200µM palmitoleate treatment in ZIKV infected cells showed cells with extensive lipid droplet accumulation but the level of lipid droplet accumulation was higher in 200µM palmitoleate treated cells when compared to 100µM of palmitoleate supplementation (Fig.4.3C-D). Treatment of 100-200µM palmitate for 48h in ZIKV infected cells showed decreased lipid droplets as well as a decreased number of cells compared to ZIKV infected cells supplemented with palmitoleate (Fig.4.3E-F).

Figure 3

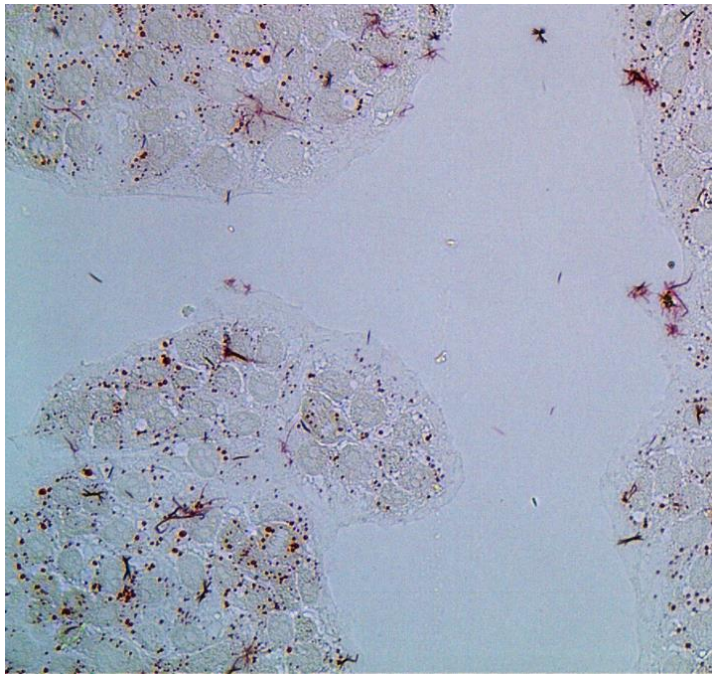
A Veh



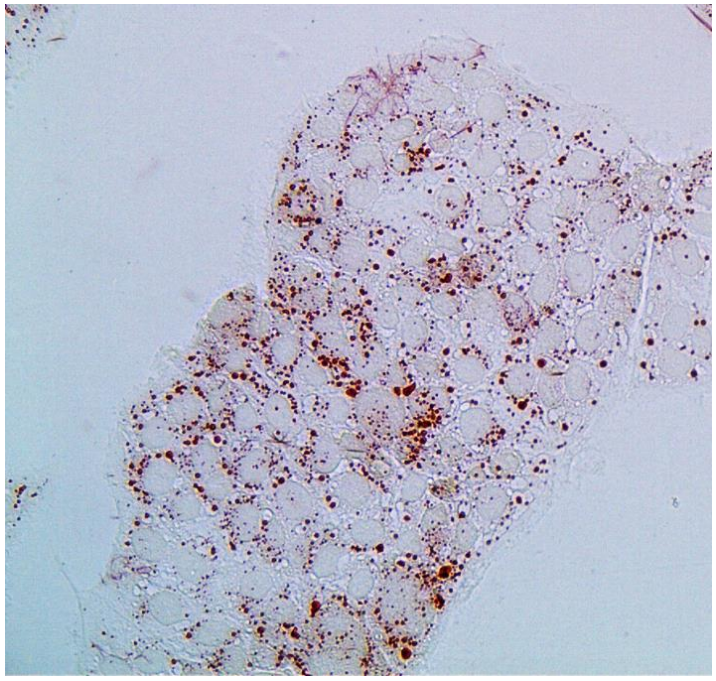
B r-MRV



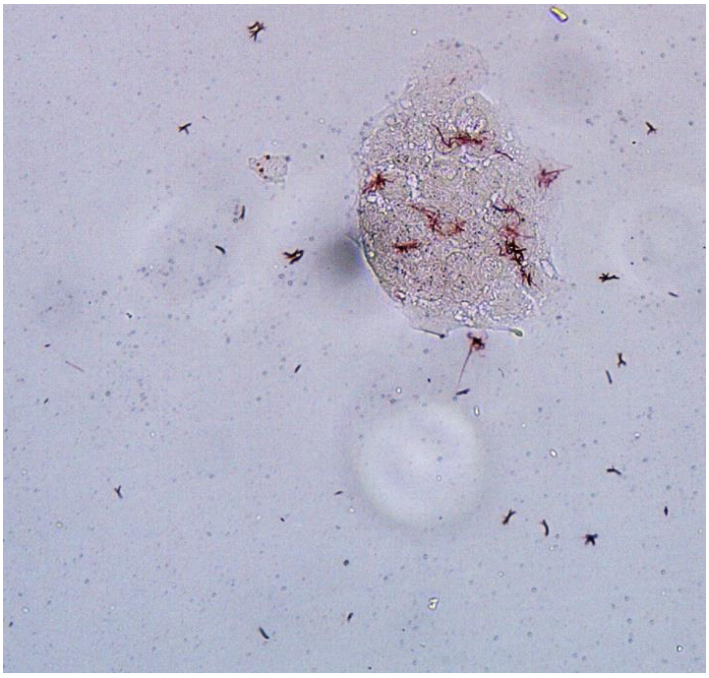
C r-MRV +100 PO



D r-MRV +200 PO



E r-MRV +100 PA



F r-MRV +200 PA

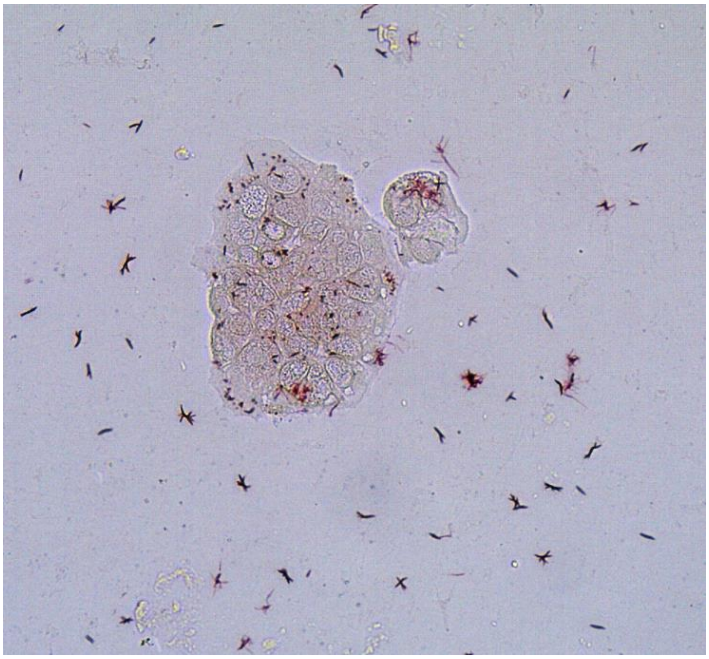


Fig.4. 3.Lipid droplet staining in ZIKV infected JEG-3 cells using oil red O staining.

Representative images after oil red O staining showing lipid droplets (A) Vehicle: Uninfected JEG-3 cells showed little or no lipid droplet accumulation. (B) r-MRV: JEG-3 cells infected with 0.1 r-MRV for 48h showed lipid droplet accumulation in the cells surrounding the infected area with cytopathic effect as shown with the arrow mark. (C) r-MRV+100 PO: JEG-3 treated with 100µM palmitoleate for 48hpi showed infected cells showed healthy cells with lipid droplet accumulation. (D) r-MRV+200 PO: JEG-3 treated with 200µM palmitoleate for 48hpi showed dramatic accumulation of lipid droplets along with a lawn of healthy cells (E-F) r-MRV+100-200µM PA: JEG-3 treated with 100-200µM palmitate for 48hpi showed fewer cells and less lipid staining when compared to ZIKV infected cells treated with palmitoleate.

4.5 Discussion

In this present study, the findings are: 1) Palmitoleate at 200µM concentration was able to reduce ZIKV viral E protein expression along with high lipid droplet accumulation 2) Oil red O staining also revealed higher lipid droplet accumulation in palmitoleate treated ZIKV infected trophoblasts when compared to palmitate treated ZIKV infected cells or ZIKV infected cells alone 3) palmitoleate treatment (200µM) significantly reduced IL-1β mRNA expression when compared to ZIKV infected cells alone.

ZIKV is known to disrupt the placental barrier and inflammation (15,16). ZIKV can increase the expression of prostaglandins associated with inflammation and 5-oxo-eicosatetraenoic, an arachidonic acid metabolite in the human placenta (3). ZIKV causes considerable alterations in the host lipid metabolism (3) in particular sphingolipids are crucial for ZIKV replication (17).

Lipid droplets apart from their conventional role of lipid storage facilitation, it is also important in innate immune signaling responses of the body (18). In our investigation of JEG-3 cells, we found that palmitoleate treatment to ZIKV infected cells resulted in profound lipid droplet accumulation and reduced viral E protein expression. This

suggests that palmitoleate could play an important protective role in activating innate immune signaling which could decrease viral E protein production. ZIKV has known to cause alterations in lipid droplets and utilize them to for their replication (18). We also noticed similar phenomena where lipid droplets were concentrated in ZIKV infected JEG-3 cells surrounding the areas showing cytopathic effect. Another study showed that ZIKV reduces the number of lipid droplets in infected Huh-7 cells (human hepatoma cell line) when compared to uninfected cells (18). In our study, we found that palmitoleate treatment for 48h in ZIKV infected cells increased lipid droplet accumulation and reduced viral E protein expression. This might be probably because the lipids trapped in the form of droplets in palmitoleate treated cells may not readily available for ZIKV to utilize for its effective replication. Lipid droplets are also known to be a protective mechanism by the host against viral infections (13).

We also noticed that IL-1β mRNA expression was significantly downregulated in 200μM palmitoleate treated JEG-3 cells infected with 0.1 MOI r-MRV. Studies have shown that palmitoleate has promising anti-inflammatory properties in human endothelial cells and high-fat diet-induced hepatitis (19,20). Decreased number of cells with palmitate supplementation in ZIKV infected cells can be related to cell death data from previous chapters. Palmitate is also known to produce an inflammatory cytokine profile in the placenta (21).

Altogether, palmitoleate treatment in ZIKV infected trophoblasts results in lipid droplet accumulation along with reduced pro-inflammatory IL-1β response. As ZIKV infection

causes IL-1β secretion by NLRP3 inflammasome activation (22), palmitoleate supplementation might also alleviate inflammasome activation in ZIKV infected cells.

4.6 Conclusion

Palmitoleate supplementation in ZIKV infected trophoblasts has a potential anti-inflammatory effect by increasing the formation of protective lipid droplets impeding viral replication and innate immune signaling.

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CHAPTER 5: SUMMARY

We confirmed that ZIKV infection with 0.1-1 MOI to JEG-3, JAR and HTR-8 cells induces apoptosis. Next, we demonstrated the activation of ER stress markers in ZIKV infected trophoblasts and found that ZIKV infection increases splicing of the *XBP1* mRNA and phosphorylation of both eiF2α and IRE1α. Further, CHOP, an ER stress biomarker and a critical mediator of apoptosis was found to translocate to the nucleus in ZIKV infected trophoblasts. In addition, JNK and p38 MAPK kinases were found to be activated via phosphorylation in JEG-3, JAR and HTR-8 cells. By using small-molecule inhibitors we have demonstrated that JNK activation is a key mediator in driving the ZIKV-induced trophoblast apoptosis.

Next, with the knowledge of the involvement of ER stress and apoptosis in ZIKV infected trophoblasts, we wanted to use a safe and protective nutrient compound that could control or prevent ZIKV spread from the infected mother to the fetus via transplacental route. We chose to use palmitoleate, an omega 7 monounsaturated fatty acid based on its cytoprotective effect against free fatty acid-induced apoptosis. We treated trophoblasts (JEG-3, JAR and HTR-8 cells) with 100-200uM palmitoleate after virus adsorption and found a reduction in the percent apoptotic nuclei and inhibition of caspase 3/7 activity, 48-72h post-infection. Further treatment of palmitoleate to ZIKV infected trophoblasts showed decreased viral E gene in the cell culture supernatant and cell lysate was reduced when compared to infected cells. To compare this unique protective property of palmitoleate we also treated trophoblasts with palmitic acid post-

infection and found that palmitic acid did not protect against ZIKV-induced apoptosis. Conversely, treatment of 200uM palmitoleate significantly downregulated CHOP expression and *XBP1* gene splicing. Even though palmitic acid-treated trophoblast supernatant had less viral titer pfu/mL, still the cell viability was considerably lower when compared to palmitoleate treated ZIKV infected cells. Taken together, from in vitro studies using trophoblasts, we found that palmitoleate treatment attenuates ZIKV-induced trophoblast apoptosis and improves cell viability with reduced viral load. We also noticed extensive lipid droplet accumulation accompanied with significant attenuation of IL-1β pro-inflammatory cytokine mRNA expression in 200μM palmitoleate treated JEG-3 cells 48hpi. Overall, palmitoleate treatment protects aganinst ZIKV infection-induced ER stress, inflammation and apoptosis.

CHAPTER 6: FUTURE DIRECTIONS

To further investigate the mechanism behind palmitoleate protection against ZIKV-induced apoptosis in trophoblasts, a transcription factor, PPARα can be studied as it is known to be involved in the positive metabolic effects induced by palmitoleate supplementation. Metabolic targets like phosphorylation of AMPK can also be studied as they are involved in the action of catabolic pathways including fatty acid β-oxidation for energy generation. Additionally studying ZIKV infection and palmitoleate treatment in Muller glial cells of the retina and testicular cells can also give some new insights.

Immunodeficient *IFNR1*$^{+/-}$ mice or immunocompetent mice with human *STAT2* gene knock-in, which can be infected with *Rag1-/-* mouse-adapted ZIKV strain can be used to study the protective effect of palmitoleate against the transplacental route of infection from the dam to the developing pups. Apart from mice models, studies in human primary trophoblasts isolated from term placental are also required to establish the protective role of palmitoleate against ZIKV infection.

In our preliminary studies, we have observed downregulation of IL-1β expression with 200μM palmitoleate treatment in ZIKV-infected cells when compared to ZIKV-infected trophoblasts alone. Investigating innate immunity pathways including NLRP3 inflammasome activation in ZIKV infected trophoblasts and the role of palmitoleate in modulating the response can also provide useful information.