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Infection by Zika Vírus in human cells alters the expression profile of miRNA-15 and activation of apoptotic caspases

Infecção por Zika Vírus em células humanas altera o perfil de expressão de miRNA-15 e ativação de caspases apoptóticas

La infección por el Vírus Zika en células humanas altera el perfil de expresión del miRNA-15 y la activación de caspasas apoptóticas

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Abstract

Objective: Evaluate the miRNA-15 expression profile involved in cellular apoptotic regulation factors. Methodology: We used the H818308 Asian strain of ZIKV without neurological damage. The inoculations occurred in human embryonic kidney cells (HEK-293). After inoculation, samples were extracted for RT-qPCR quantification of viral RNA and miR-15. The level of activation of caspases 1, 3/7 and 8 of cells was performed using chemofluorescence. Results: The ZIKV infection alters the expression of genes and their regulators, affecting several cellular physiological processes such as apoptosis. Conclusion: Therefore, it is important to emphasize that renal progenitor cells (HEK-293) are susceptible to VZIK infection. The genetic deregulation resulting from infection directly affects important cellular processes such as apoptosis from the disordered miRNA-15 expression during the infection period.

Keywords: Zika virus; Apoptosis; miRNA-15; HEK-293.

Resumo

Objetivo: Avaliar o perfil de expressão do miRNA-15 envolvido nos fatores de regulação da apoptose celular. Metodologia: Usamos a cepa asiática H818308 do ZIKV sem danos

neurológicos. As inoculações ocorreram em células renais embrionárias humanas (HEK-293). Após a inoculação, as amostras foram extraídas para quantificação RT-qPCR do RNA viral e miR-15. O nível de ativação das caspases 1, 3/7 e 8 das células foi realizado por quimofluorescência. Resultados: A infecção pelo ZIKV altera a expressão de genes e seus reguladores, afetando diversos processos fisiológicos celulares, como a apoptose. Conclusão: Portanto, é importante enfatizar que as células progenitoras renais (HEK-293) são suscetíveis à infecção por VZIK. A desregulação genética resultante da infecção afeta diretamente processos celulares importantes, como a apoptose da expressão desordenada do miRNA-15 durante o período da infecção.

Palavras-chave: Zika virus; Apoptose; miR-15; HEK-293.

Resumen

Objetivo: Evaluar el perfil de expresión de miARN-15 implicado en factores de regulación apoptótica celular. Metodología: Utilizamos la cepa asiática H818308 de ZIKV sin daño neurológico. Las inoculaciones se produjeron en células renales embrionarias humanas (HEK-293). Después de la inoculación, se extrajeron muestras para la cuantificación RT-qPCR de ARN viral y miR-15. El nivel de activación de las caspasas 1, 3/7 y 8 de las células se realizó mediante quimiofluorescencia. Resultados: La infección por ZIKV altera la expresión de genes y sus reguladores, afectando varios procesos fisiológicos celulares como la apoptosis. Conclusión: Por lo tanto, es importante enfatizar que las células progenitoras renales (HEK-293) son susceptibles a la infección por VZIK. La desregulación genética resultante de la infección afecta directamente a importantes procesos celulares como la apoptosis de la expresión alterada del miARN-15 durante el período de infección.

Palabras clave: Virus Zika; Apoptosis; miRNA-15; HEK-293.

1. Introduction

The Zika virus (ZIKV) is an arbovirus classified in *Flaviviridae* family, genus *Flavivirus*. It was first isolated in 1947 in the Zika forest, Uganda, from a non-human primate that served as a sentinel for studying the Yellow Fever Virus (VFA). Like most arboviruses, VZIK needs a hematophagous arthropod vector to maintain its cycle of replication and dissemination, today is an emerging pathogen with a broad seasonal spectrum and epidemiological relevance worldwide, due to the ability to cause different pathological conditions. in newborns and adults. (Amorim, et al, 2020; Who, 2009; Dick, et al,, 1952; Duffy,

et al., 2009).

The ZIKV has a genome consisting of single-stranded positive polarity RNA, containing information for the coding structural and non-structural proteins, which has the purpose of favoring viral replication by inhibiting the defense mechanisms of its host. The virus-host relationship directly interferes with the expression of many immune response genes, causing modifications in their expression levels. The mediators of gene expression are non-coding protein RNAs, such as microRNAs (miRNA), which act at the post-transcriptional level by controlling the expression of genes from the degradation or repression of the translation of mRNA target molecules (Cowland, et al, 2007; Chambers, et al, 1990).

Among the molecules that show a difference in the cellular response during viral infection are microRNAs, which are a group of small non-coding RNAs, whose functions are related during the regulation of a diverse variety of cellular processes such as differentiation, growth, proliferation, migration, apoptosis, metabolism and immune response (Bartel, 2004; Carvalho, et al, 2011). These miRNAs can originate through several pathways, the most common is the canonical pathway, which involves the transcription of the miRNA from the coding gene, its maturation and incorporation into the RNA-induced silencing complex (RISC, RNA-induced silencing complex) (Kim, et al, 2009).

These molecules work by directly linking to the 3' untranslated regions (3'UTRs) of the specific target mRNA, causing decrease translation or degradation of the target mRNA (Garofalo, et al, 2008). Thus, through the regulation of genes, miRNAs specifically become involved in important cellular physiological processes. (Pauley & Chan, 2008; O'connell, et al, 2012; Azouz, et al, 2019). Despite the relative nature of the functionality of viral miRNAs, it is still emerging in view of the establishment of their influence at the cellular level, it is noted that these molecules may be involved in cellular reprogramming in order to: 1) regulate the lytic latent keys; 2) support viral replication, promoting cells to proliferation and/or differentiation and; 3) modulate immune responses (Bartel, 2009; Skalsky & Cullen, 2010). In this perspective, the integration of the host cell is promoted by multiple and redundant mechanisms, with miRNAs and viral proteins acting synergistically in order to provide a conditionally favorable environment for the conclusion of the viral cycle (Skalsky & Cullen, 2010).

Studies suggest that the differentiated expression of miRNAs has a substantial relationship under a crucial regulatory role in neurodegenerative diseases (Junn & Mouradian, 2012). Other studies describe the alteration in the miRNAs cellular profile in response to viral infection, as a result of the cell's innate immune response to inhibit virus replication or viral strategy (Souza, 2016; Umbach & Cullen, 2009). In addition, studies highlight the differential

expression of cellular miRNAs, closely related to viral infection, both in vertebrates and invertebrates, indicating that some viruses might have the ability to deregulate the miRNA profile. Thus, there is a variation in expression levels of these miRNAs, resulting from the host's immune response to viral infection or by the interference of the viral replication cycle in the host cell (Asgary, 2014). It is also established the relationship of miRNAs in apoptosis, as well as in studies that promote the debate on the interaction in metabolism and cellular defense (Pfeffer, 2007). In this perspective, some specific miRNAs, such as mIR-15, may undergo changes in their expression levels during viral infection, since they are involved in the host's immune response, making it essential to study their expression and the cellular responses, such as apoptosis (Kozaki et al, 2008; Umbach & Cullen, 2009).

Apoptosis, or programmed cell death, is an essential physiological process for cell maintenance, although this mechanism is also involved in pathological conditions, as seen in viral infections (Luchs & Pantaleão, 2010). The main effector proteins activated during apoptosis are caspases, or aspartate-specific cysteine-dependent proteases, whose function is to cleave cellular substrates, resulting in the amplification of the apoptotic process and the beginning cell breakdown (Cuconati & White, 2002). In order to occur, the target protein must have a caspase cleavage site containing aspartic acid residue, in addition to being recognized by the cysteine side chain enzyme that catalyzes the cleavage of the peptide bond. Caspases can exist in the cell in inactive pro-caspases or zymogens, cleaved and activated in response to apoptotic stimuli, activated via a proteolytic cascade (apoptotic caspases), or as caspase initiators (activated first, containing a longer pro-domain - caspases 2, 8, 9 and 10) (Cuconati & White, 2002; Best & Bloom, 2003). These caspases, cleave and activate a shorter pro-domain, called effector caspases (caspases 3, 6 and 7). Caspase 3 cleaves most cell substrates during apoptosis and is also responsible for cleaving viral proteins. Caspases 7, 6 and 2 are also important in the cleavage of viral protein substrates (Ricarte & Kimura, 2006).

In this perspective, several discoveries related to ZIKV tropism by neural progenitor cells infer the susceptibility of an apoptosis trigger, which, in a systematic spectrum, introduces the ability to contribute to a neurological impact, although it is possible to establish a direct relationship the tropic multiplicity of VZIK with cells from different tissues (Dang, et al, 2016; Tang, et al, 2016; Miner, Diamond, 2017). Human studies have detected viral RNA in maternal and fetal tissue, originating in the placental matrix, umbilical cord, amniotic fluid and during the fetal and neonatal development of the human brain, thus revealing a broad tissue tropism by progenitor cells (Onorati, et al, 2016; Garcez et al, 2016). It is then noted, in function analogous to tissues, the need for studies that promote the search for analysis of apoptotic

activation in renal progenitor cells infected by VZIK, in order to demonstrate significant results in embryonic kidney cells (Miner & Diamond, 2017) .

2. Methodology

Experimental, cross-sectional, quantitative study (Pereira, 2018). The strain used in this work is registered H818308 and is stored in cell culture of *Aedes albopictus* (Clone C6 / 36), stored at -70ºC, belonging to the Section of Arbovirology and Hemorrhagic Fevers of the Evandro Chagas Institute. The original sample comes from a patient who died of ZIKV infection in the state of Maranhão (Brazil).

2.1 Viral stock in *Aedes albopictus* cell culture, clone C6 / 36

The viral stock was obtained from the inoculation and cell culture of *Aedes albopictus* clone C6 / 36 cells. For the maintenance of cell culture, the L-15 medium was used, keeping the cells at 28ºC with weekly passages of the confluent monolayers in 25cm² culture bottles containing 10mL of growth medium. Subsequently, the cells were transferred to 175 cm² bottles after the formation of the monolayer, approximately on the third day, the cell cultures were subjected to infection in the proportion of 1: 100. On the tenth day after inoculation, aliquots of infected cell suspension were collected for the indirect immunofluorescence test (IFI). Subsequently, the stock was stored at -70oC until the moment of use.

2.2 Inoculation in HEK-293 cells

HEK-293 cells (human embryonic kidney) were inoculated into 6-well cell culture plates in biological triplicate, with 5 wells from each plate corresponding to the Days Post-Infection (DPI) and the remaining wells became negative infection controls (MOCK). Each plate well represents about 9.20 cm² filled with DMEM (Dulbecco's Modified Eagle's) medium enriched with 10% Bovine Fetal Serum (SBF). For inoculation, the value of 0.5 was used for Multiplicity of Infection (MOI) and the method of adsorption for one hour at 37 ° C. After virus adsorption, cells were washed with 1x buffered saline (PBS) 1x, placed in DMEM maintenance culture medium with 2% SBF and cultured for further analysis. One plate well was collected every 24 hours post-infection, totaling five collections.

2.3 Extraction of cellular miRNA and viral RNA

As amostras foram extraídas na plataforma Maxwell (Promega, EUA) utilizando os kits comerciais *Maxwell 16 miRNA Tissue Kit* (Promega, EUA) para extração de RNA total celular e *Maxwell 16 Lev Viral Total* (Promega, EUA) para extração de RNA viral a partir do sobrenadante de células infectadas para quantificação de carga viral, ambos seguindo os protocolos de acordo com o fabricante.

For extraction of total cellular RNA on Maxwell platform (Promega, USA) it was used the commercial kits Maxwell 16 miRNA Tissue Kit (Promega, USA), and for extraction of viral RNA, it was used the Maxwell 16 Lev Viral Total (Promega, USA) from of the supernatant of infected cells for quantification of viral load, both following the protocols according to the manufacturer.

2.4 Quantification of miRNA 15 and viral load by RT-qPCR

The RT-qPCR processing, the commercial kit GoTaq 2-Step RT-qPCR System (Promega, USA) was used in the first step, the Reverse Transcription (RT) step using Random primes and GoScript (Promega, USA) as described by manufacturer. Then, a quantitative PCR was performed using the Rotor-Gene Q platform (Qiagen, USA). For this step, primers and probes for the target miRNA-15 (UAGCAGCACAUAAUGGUUUGUG) were used, this miRNA being involved in the process of cell apoptosis. The relative amounts of target miRNA in the sample were calculated and normalized to the corresponding level of the transcribed miRNA RNU 46 and RNU 20 (endogenous controls) and in the analysis the 2^{-ddCt} comparative method was used (Livak & Schimitgen, 2001).

Quantification of viral load was performed on the Rotor-Gene Q platform (Qiagen, USA) using the *SuperScript III Platinum One-Step RT-qPCR* kit (Invitrogen, USA) according to the manufacturer's specifications. The quantification method was the same as described by Duffy, et al (2009) and with the use of a plasmid for absolute quantification.

2.5 Activation of apoptotic caspases detection

To detect caspase activation in infected and MOCK cells, the GlomaxMulti+ platform (Promega, USA) was used through the flowering method according to the manufacturer's

specifications. The caspases that were determined as a target are related to both the extrinsic and intrinsic pathways of apoptosis and are listed in Table 1.

Table 1. Caspases Kits – GlomaxMulti+ platform (Promega, USA).

Target	Kit
Caspase 1	**Caspase-Glo 1 Assay**
Caspase 3/7	**Caspase-Glo 3/7 Assay**
Caspase 8	**Caspase-Glo 8 Assay**

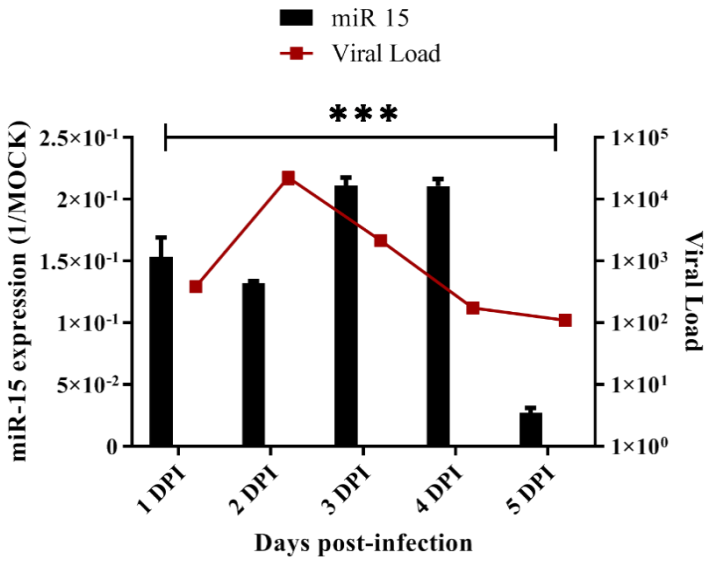
Source: Authores (2020).

The data were exported for statistical analysis using Expression Suite v1.0 (AppliedBiosystem, USA) and the R Project program (r-project.org) for statistical analysis. Samples infected with ZIKV were compared with uninfected cells and analyzed using the ANOVA test.

3. Resullts

The results obtained are consistent with several studies available in the bibliographic databases related to gene disorders resulting from viral infections. According to the research products, it was possible to establish that ZIKV infection HEK-293 cells is possible, since this cell line has been shown to be susceptible and permissible to infection, undergoing changes in expression regulators and resulting in the interference of important mediators of apoptosis. The (Figure 1**)** shows that there is a considerable number of copies of viral genome at different periods of infection, resulting in the miR-15 expression disorder.

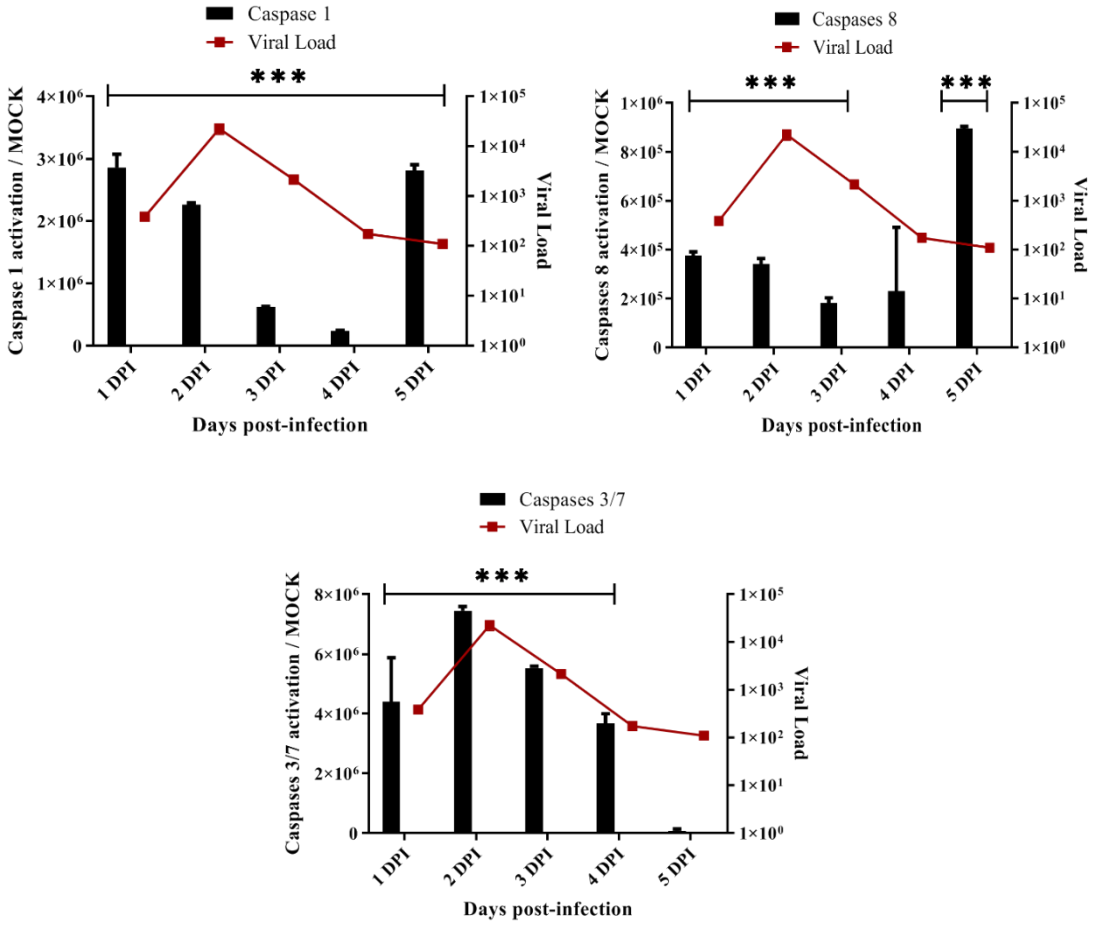
Figure 1. Quantification of viral load and miR-15 expression during VZIK infection in HEK-293 cells.



Source: Authors (2020).

Graphical representation of miR-15 expression and viral load during days 1, 2, 3, 4, and 5 after ZIKV infection in HEK-293 cells. The value of *p <0.05 (***)* was considered to determine the statistical significance of the results. The viral load changes during the infection period, with its peak at 2 DPI and 3 DPI respectively. However, there is a significant decline from the 3 DPI determining the final stage in the infection period. It is noticeable that the infection caused by ZIKV alters the level of expression of miR-15, this microRNA aims to regulate genes involved in apoptosis activation. MiR-15 is positively expressed during the infection period and has its peak expression between 3 DPI and 4 DPI, and may be closely related to viral load, as it is suggestive that the host needs apoptotic activation to ease ZIKV infection, resulting in the disordered miR-15 expression. The 5 DPI shows that with the decrease in viral load, there is also a considerable decline in the expression of miR-15, determining that infection by ZIKV causes changes in apoptotic gene regulators according to their level of replication in the host. Furthermore, it was related to ZIKV infection with the activation of pro-apoptotic and effector caspases during the infection period, which are mediators of the activation of the apoptosis process by extrinsic and intrinsic via the chemofluorescence method as shown in Figure 2.

Figure 2. Caspases Activation during ZIKV infection in HEK-293 cells.



Source: Authors (2020).

Correlation between viral load and activation of caspases during infection by ZIKV in HEK-293 cells: (A) Caspase 1 activation and viral load; (B) Caspase 8 activation and viral load; (C) Caspases 3/7 activation and viral load; All graphs show statistical significance considering the value of $p < 0.05$ (***).

It is observed that infection by ZIKV in HEK-293 cells is also responsible for causing the change in caspase activity, which are mediators of apoptosis and inflammasome, such as caspase-1. It is possible to observe in graph A, that caspase-1 is less active during 3DPI and 4DPI, with an apparent decline from 2 DPI, a phase where the highest viremia peak occurs, and although the values corroborate and have overlapping parallelism between 2-4 DPI. The demonstrated products suggest that the infection may interfere by inhibiting the action of this caspase to maintain the viral replication cycle, since caspase-1 it is also related to inflammasome, could signal the activation of inflammatory cytokines and generate a more accentuated response to infection.

Caspase-8 (Figure 2 - B) has been shown to be inactive during most of the infection period, despite the peak of viremia demonstrated from 2 DPI with subsequent decline. Based on the 5 DPI super expression, it is possible to determine that apoptosis is one of the most used response mechanisms against ZIKV infection, due to the measured results indicating that the infection alters both post-transcriptional gene expression to the process mediators the effectiveness of host responses to ZIKV infection in HEK-293 cells.

Caspases-3/7 (Figure 1 - C) are mediators of the extrinsic pathway of apoptosis and classified as effector. These showed great activation during the 1,2,3 and 4 DPIs. However, at 5 DPI the 3/7 caspases were not very active and this may have been influenced by the effectiveness of the cell death process, resulting in the low activation of these caspases and also in the decrease of the viral load in the host. In this perspective, the exposed data show that ZIKV infection can activate the two pathways of cell death and that this mechanism is one of the most used by the host in response to infection, as it is related to the decline in viral load.

4. Discussion

Over the years, ZIKV has become a matter of public disorder due to its ability to foment crises in the economic and social spectrum. The ineffectiveness of public policies in the control of the vector has increased the number of cases of the disease in regions of dominant endemicity, concomitantly associated with the proliferation of the agent. Thus, strategies that promote the search for host immune response mechanisms associated with the biogenesis of molecules that mediate gene expression become more effective, as they were able to establish the physiological basis of the human cells behavior against viral infection (Amorim, et al, 2020; Fecury, et al, 2020).

This arbovirus, stood out mainly for its ability to cause mild symptoms such as fever and headache, even the most severe neurological cases (Chambers, et al, 1990). Although the infection occurs both in adults and in newborns, ZIKV has a preference for progenitor cells, in this study, it was possible to observe that HEK-293 cells are susceptible and permissible to viral infection, corroborating the findings of Chen, et al (2017), who detected the presence of ZIKV in renal cells and cytopathic effects, such as caspase 3-mediated apoptosis, resulting from infection in mice.

Milner, et al (2017), also demonstrated that ZIKV has tropism related to cells of several progenitor strains, some of which originated from the placenta, trophoblasts and endothelial cells. The same study that mentions others ZIKV cellular targets, such as neuronal cells,

including neural progenitor cells, mature neurons and astrocytes, describes the ZIKV tropism, being responsible for the detection of the virus in several human cells, originating from the most different origins of development, as observed in the detection of body fluids, such as semen, saliva, cervical mucus and urine, thus elucidating that the urinary system can viral load in the period of infection carried out in our study.

Arboviruses have tropism for different cells and may present similar peaks of infection and cause various cytopathic effects. Casseb, et al (2017) showed that the peak of infection by DENV-4 in A549 cells occurs at 3DPI and 4 DPI, and that infection by this serotype of DENV also caused the disordered expression of genes, affecting the mediators of the canonical pathway in biogenesis of miRNAs. Thus, it is possible to infer that several arboviruses use cellular or proprietary miRNAs in order to direct cellular and viral processes, in order to promote, establish and conclude their replicative process, Ferreira, et al (2019) showed in analysis of mediated gene expression by non-coding RNAs in *Mesocricetus auratus* infected with ZIKV

As observed in Holanda, et al (2017), the infection by the two genotypes of YFV in HepG2 cells also caused the disordered alteration in the expression of genes involved in the biogenesis of miRNAs through the DROSHA, Ago2, DGCR8 and Ago4 mediators present in the canonical way. Ferreira, et al (2018) also related ZIKV infection to the dysregulation of these genes in HepG2, A549 and MA104 cells. Thus, according to the mentioned studies, under analysis of the results obtained, it is possible to establish the contribution with our findings in the deregulation of miR-15 during the infection period, as well as the relation of arbovirus infection in the host's immunogenetics.

Studies demonstrate that several *Flaviviruses* such as DENV, YFV and Japanese Encephalitis Virus are related to miR-21-5p and miR146a-5p, that are involved with cell proliferation and inflammatory processes during infection. These same authors also describe that miRNA-15 and 16 may have an increased expression during infection by DENV and that this may be related to the NS1 protein and the loss of viability and cell death (Casseb & Melo, 2020). Although our study is not related to the NS1 protein of ZIKV, it is possible to notice that the miR-15 pattern is similar between DENV and ZIKV expression.

Hutvagner (2005), shows that miRNAs are closely related to gene expression, and can act by silencing target genes, which makes this theory important for maintaining the viral replication cycle. Fecury, et al (2020), demonstrated that ZIKV infection alters all mediators of miRNA biogenesis and that this directly affects the host's immune response to infection. Our study demonstrates that miR-15 undergoes significant changes in its level of expression during

ZIKV infection, which is an important regulator of apoptosis, and it may be suggestive that the virus uses this immunogenetic mechanism to evade the immune response (Zhang, et al, 2009). Therefore, since apoptosis is one of the mechanisms used by the host to mitigate the infection by ZIKV, the activation of caspases was observed during the infection period and we noticed that the virus triggers activation by different apoptotic pathways and that the derangement of caspases is related with the infection. According to Best & Bloom (2003), viruses encode caspase activity inhibitors to evade apoptosis, and thus prolong their replication cycle.

Martin & Cullen (2009), demonstrate that caspases are pro-apoptotic and effector proteases, which can be activated intrinsically and extrinsically, their exacerbation can influence the activation of inflammasome and release of interleukins. As also shown by Wong, et al (2008), there is an induction of inflammatory response during infection by ZIKV through the release of cytokines by the host. Thus, we could also observe in our findings that there is significant deregulation of caspases during the period of infection by ZIKV. In addition, Souza, et al (2016), also signified several cytopathic effects in their studies, resulting from ZIKV infection through immunohistochemistry, where great activation of caspases was found and, consequently, activation of apoptosis, release of different cytokines during the infection period. It is important to note that our results contribute to these studies, since we can observe these changes at the molecular level.

Several ZIKV replication studies in human cells have shown that during the viral infection process, several metabolic pathways are altered, whether to favor the host to develop a response or to maintain the viral replication cycle. Thus, basic research that seeks to understand the miRNA biogenesis resulting from ZIKV infection is essential in order to relate to the cell's response mechanisms. As well as, more in-depth studies involving molecular techniques that help to determine the host's immunogenetic factors, thus being able to clarify the virus-host relationship in order to find efficient and effective therapeutic measures against ZIKV infection.

5. Conclusion

This study made it possible to relate the expression of miRNA 15 and apoptosis pathways during VZIK infection, based on the exploration of the host's defense mechanisms against infection. Thus, it was possible to observe that the viral load reaches its peak on the third day of infection and the high activation of caspases is related to the second day after

infection, although it is likely that ZIKV infection is responsible for activating the two pathways of apoptosis.

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