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Apolipoproteins and Its Association with Dengue Virus Serotype 2 (DENV-2) Infectivity in Human Hepatoma Cell Line (Huh7)

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Abstract

Introduction: Previous studies have shown that lipid components are involved in the internalization of Dengue virus (DENV), however, this mechanism has been hypothesized mostly through clinical studies correlating between dengue severity and lipid levels. This study aimed to determine DENV-2 internalization in the presence of apolipoprotein A1 (apoA1), B (apoB) or E (apoE) in human hepatoma cell line (Huh7). *Materials and methods*: Tetrazolium reduction assays were used to assess the cytotoxicity of apolipoproteins on Huh7. DENV-2 at MOI of 1 with 2 µg/mL apoA1, apoB and apoE, respectively were introduced into the confluent Huh7 cells and incubated for an hour (internalization) and 72 h (replication) at 37 °C, 5 % CO_2. The replication experiments were repeated after SR-B1 siRNA treatment. Samples collected were subjected to qPCR for viral load determination. Differences between groups were assessed by ANOVA and independent T-Test. *Results and discussion*: Treatment with apoA1 and apoB demonstrated significant increment in DENV-2 internalization and replication compared to apoE (internalization: $p = 0.022$ and $p = 0.323$ vs $p = 0.878$, respectively; replication: $p = 0.004$ and $p = 0.016$ vs $p = 0.897$, respectively). A significant reduction of DENV2 infectivity was seen in apoA1 treated cells with SR-B1 down-regulation/silencing (35.2 % reduction, $p = 0.013$). *Conclusions*: apoA1 and apoB, enhanced initial attachment of DENV-2 to cell surface, suggesting their role in DENV internalization and infectivity.

Keywords: Dengue, Apolipoprotein, apoA1, apoB, apoE

Introduction

Dengue fever is one of the most prevalent mosquito-borne diseases caused by dengue virus (DENV) that is transmitted by the Aedes female mosquitoes, principally *Aedes aegypti* [1]. The infection produces a wide spectrum of manifestations, ranging from sudden onset of high-grade fever, nausea, vomiting, severe headache, muscle and joint pains, and a characteristic skin rash [1]. However, in some cases, it can lead to life-threatening complications, including dengue haemorrhagic fever and dengue shock syndrome

[2,3]. Geographically, it occurs in tropical and subtropical areas of the world especially in urban and semi-urban areas [4].

Over the past 2 decades, there has been a dramatic increase in dengue cases reported to the World Health Organization (WHO), from 505,430 dengue cases in 2000 to over 2.4 million in 2010, and 5.2 million in 2019 [5]. Between 2000 and 2015, the number of reported deaths increased over 4 fold from 960 to 4,032 deaths, dominated by younger age groups [5]. It is estimated that 3.9 billion people are at risk of dengue in 128 countries [6]. According to the WHO (2022), the disease is currently endemic in more than 100 countries where the Americas, South-East Asia, and Western Pacific are the most severely impacted regions, with Asia accounting for approximately 3-quarter of the global burden of disease [5]. In Malaysia, dengue reported cases and deaths have risen dramatically from 43,346 cases with 92 deaths to 108,698 with 215 deaths once laboratory confirmation tests such as NS1/ IgM/ IgG/ PCR/ viral isolation are mandatory for case registration starting in 2014 [7]. Annual cases of dengue remain at an alarming rate from 2014 to 2020 with an average value of 102,251 cases and 205 deaths [7].

DENV is a single positive-stranded RNA virus of the family Flaviviridae; the genus Flavivirus is divided into 4 serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) based on its genetically and antigenically distinction [2]. Infection with 1 type usually provides lifelong but specific immunity to that type only, and rendering short-term immunity to the others [8]. Subsequent infection with a different serotype increases the risk of severe complications [8]. Viral genetic components determine virulence and infectious potential. Thus, DENV serotypes' structural peculiarities have an impact on pathogenesis. Serotypes with a tendency to have faster rates of replication cause a faster generation of antibodies, leading to more severe effects. DENV-2 has been classified as the most virulent serotype due to its association with more frequent epidemics and severe manifestations. Vaughn *et al.* [9] discovered that secondary DENV-2 induced more severe complications than other serotypes. A cross-sectional investigation of 485 confirmed dengue cases by Vicente *et al*. [10] discovered that cases of DENV-2 had a higher proportion of severe dengue than cases of DENV-1 and DENV-4.

DENV enters host cells through receptor-mediated endocytosis and the mechanism of DENV internalization comprises a number of phases and interactions between the virus and the host cell. Adhesion or viral attachment to the cell surface is the initial stage of DENV penetration where the viral envelope glycoprotein E binds to cellular receptors such as DC-SIGN, a C-type lectin or heparan sulfate proteoglycans [11,12]. Followed by endocytosis through clathrin-mediated endocytosis [13]. Then the viral envelope glycoprotein E undergoes conformational change due to low pH environment causing the viral and endosomal membranes to fuse and release the viral genome into the cytoplasm. Viral proteins and RNA are produced as a result of the translation and replication of the viral genome by the host cells. Finally, the viral proteins and RNA are assembled to form new virus particles, which are then discharged and infect other host cells. DENV internalization is a complex process and understanding the mechanisms of dengue entry is important for DENV treatment and intervention.

Previous studies have reported that virus infectivity is related to plasma cholesterol and lipoprotein concentrations [14]. Several clinical studies have shown the association between high density lipoprotein cholesterol (HDL-c) and low density lipoprotein cholesterol (LDL-c) levels with DENV infection [15,16]. Apolipoprotein A1 (apoA1), a major component of HDL-c was shown to have an association with dengue severity [17]. Previous studies have found that LDL-c concentrations at presentation were associated with subsequent risk of developing dengue hemorrhagic fever [15]. Therefore, studies on the effects of HDL-c and LDL-c components on dengue infectivity are essential as part of a prognostic biomarkers panel to identify potential patients who may develop severe dengue. It is also noted that predicting severe dengue is difficult due to the wide spectrum of the disease and the fact that not all dengue cases progress to severe form. At the moment, the predictive factor for severe dengue is based on a combination of clinical assessment, laboratory tests, and observation of specific warning signs which is time consuming and may cause a delay to provide appropriate treatment to the patient. Since previous studies have found the involvement of HDL-c and LDL-c in modulating dengue infectivity, measuring both cholesterols during early infection could potentially predict the likelihood of severe dengue which allows for earlier intervention to avoid it. It can act as a screening method, allowing early stratification of the patient and providing ample time in managing and reducing the severity of dengue infections.

Recently, several studies have reported on the role of scavenger receptor class B type 1 (SR-B1) receptors as one of the potential routes of entry for the DENV into the cell [17,18]. SR-B1 is involved in the uptake of HDL-c and other lipoprotein fragments from peripheral tissues and transports them to the liver for excretion. Since viruses require cholesterol for viral entry, transportation and protection during the infection, the SR-B1 receptors are perfect targets for the DENV. This present study aims to provide further insight into the role human apoA1, apoB and apoE have in dengue internalization and replication

in the presence and absence of SR-B1 receptors. Findings from this study could point us in the direction of better early intervention of dengue infections and possible newer therapeutic channels via the lipid pathway that can help with dengue management.

Materials and methods

Propagation of dengue virus serotype 2 (DEN2)

The DENV-2 (VR-1584, New Guinea C, 1944) was propagated in C6/36 Aedes albopictus cells grown in Leibovitz's L-15 Medium supplemented with 1 % fetal bovine serum (FBS) and 10 % tryptone phosphate broth. The infected C6/36 was incubated at 27 °C without CO_2 until 80 - 90 % cytopathic effect was observed. The supernatant then was harvested and clarified by centrifugation at 2,000 rpm for 15 min to generate virus stock. Two forms of DENV-2 stocks were prepared: (1) DENV-2 in serum free media (DENV-2/SFM). (2) DENV-2/SFM was added with either human apolipoprotein A1, B or E generating DENV-2/ApoA1, DENV-2/apoB and DENV-2/apoE.

Cytotoxicity testing of apolipoproteins on Huh7 cell line

The Huh7 cells (5×10^3 cells/well) were seeded into 96 clear, flat-bottomed wells of microtiter plates (Nunc, Denmark) containing Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 % FBS and 1 % antibiotics. The microtiter plates were incubated at 37 °C, 5 % CO_2 overnight and proceeded with cytotoxicity testing to determine cell viability of Huh7 cells against apolipoproteins. The media was removed and the cells were treated with 100 µL of various concentrations of each apolipoprotein (apoA1/apoB/apoE) ranging from 0.25 to 2.00 µg/mL. Untreated cells were included as control wells. The microtiter plate was then incubated for an hour at 37 °C, 5 % CO_2. The 20 µL MTS solution was added to each well and incubated for another hour at 37 °C. The absorbance was measured at 490 nm using Victor X52030 Multilabel Plate reader (Perkin Elmer, USA) and the viability of the cells was measured by comparing the treated wells with the control wells.

Effects of apolipoproteins on virus binding and internalization

The Huh7 cells were seeded in 24-well microtiter plate and infected with DENV-2 by incubating the cells with DENV-2/apoAI, DENV-2/apoB and DENV-2/apoE at MOI of 1, respectively for an hour. DENV-2/SFM without the presence of any apolipoproteins was included as control and labeled as a non-treated sample. Virus bindings were measured directly after 1 h of incubation. The cells were washed with PBS, lysed with a lysis buffer and subjected to viral load measurement via qPCR. For confirmation, the cells were incubated for 72 h to allow virus replication. Supernatant was collected and subjected to viral RNA extraction and viral load measurement via qPCR.

Silencing of the scavenger receptor Class B Type 1 (SR-B1)

SR-B1 silencing was done in separate experiments. About 6.0×10^4 cells/well Huh7 were seeded in 24-well plates and incubated at 37 °C, 5 % CO_2 overnight. The cells were transfected with siRNA followed by 4 h of incubation. For silencing validation, negative control was included using Allstars Negative siRNA (Qiagen, Germany), a non-targeting siRNA to detect non-specific effects that might occur during the process of siRNA introduction into the cells. The media was changed and incubated for another 48 h. Subsequently, the cells were incubated with DENV-2/apoAI, DENV-2/apoB and DENV-2/apoE at MOI of 1, respectively for an hour. DENV-2/SFM was included as control and labeled as a non-treated sample. The cells were washed once with PBS and incubated for 72 h. The supernatant was subjected to viral RNA extraction and qPCR analysis.

RNA extraction and qPCR assay

Culture supernatants were collected from DENV-treated Huh7 cells and subjected to total RNA extraction using NucleoSpin RNA Plus in accordance with the manufacturer's protocol. Viral loads were measured using dengue specific primer and amplification will be carried out in BIO-RAD CFX96 using Genesig Standard Real-time PCR detection kit for Dengue Virus as described in the manufacturer's instructions. Cq (quantification cycle) results obtained were converted to RNA copy numbers. Standard curves for DENV RNA copy number determination were constructed using Genesig Standard Real-time PCR detection kit for Dengue Virus (Primer Design, UK) in accordance with the manufacturer's instructions.

Statistical analysis

DENV RNA copy numbers were presented as fold change. Fold change values were obtained by normalizing the experimental data against the control data (non-treated sample) for relative comparison. Cytotoxicity testing was assessed using ANOVA. Differences of viral load between apoA1, apoB, apoE and non-treated samples were assessed by ANOVA and pairwise differences of viral load between siRNA treatment and non-silent cells determined by independent T-Test with SPSS for Windows (version 20, USA). All data were expressed as mean ± SD. The level of statistical significance was at $p < 0.05$.

Results and discussion

It has been well established that lipid profile changes will inevitably be identified with increasing dengue severity. Gorp *et al.* [19] reported that levels of total plasma cholesterol, HDL-c and LDL-c were significantly decreased in patients with severe DENV infection, compared to patients with mild disease and healthy individuals. This finding was supported by a prospective study conducted by Biswas *et al.* [15] which reported that decrease in serum cholesterol and LDL-c levels were observed among severe dengue patients compared to mild dengue cases. Similarly, Chuck [20] reported a decrease in total cholesterol and LDL-c levels with increasing dengue severity. However, the specific mechanism and relationship between development of severe dengue with total cholesterol, HDL-c and LDL-c, respectively, remain unclear. The involvement of HDL-c and LDL-c in DENV infection suggests apolipoproteins, the major components of HDL-c and LDL-c, may play a role in dengue infectivity.

In this study, 3 apolipoproteins of interest, apoA1, apoB and apoE were applied to identify its modulating effects on DENV-2 internalization into the Huh7 cell line. Those apolipoproteins were selected based on its structure as the major structural and functional protein components of HDL-c and LDL-c and its interaction with SR-B1 receptors which are located at the target organ for DENV-2. It is well established that apolipoproteins are involved in assisting Hepatitis C virus (HCV) internalization into the target cells and may have the same effects on DENV infectivity since HCV and DENV belong to the same family which share many virulence features. The Huh7 cell line represents the target organ and was chosen due to its high susceptibility to virus infection, allowing efficient production of infectious virus particles *in vitro* and it is the most suitable model for investigating DENV infectivity [21].

Cytotoxicity testing of apolipoproteins on Huh7 cell line

Prior to conducting the internalization assay, effects of apoA1, apoB and apoE on Huh7 cells viability were measured in order to avoid cytotoxic effects of each apolipoproteins on the cells. **Figure 1** shows the cell viability of Huh7 after being treated with different concentrations of apoA1, apoB and apoE ranging from 0.25 to 2.00 µg/mL. There were no significance difference between apoA1 and apoB treated cells compared to control cells across all concentration, while apoE exhibit significance reduction at high concentrations (1.00 µg/mL ($p = 0.029$), 2.00 µg/mL ($p = 0.027$)). However, despite the difference, cell viability remained above 80 % at this concentration and according to ISO 10993-5 (Tests for *in vitro* cytotoxicity), percentages of cell viability above 80 % are considered as non-cytotoxicity [22]. Since all apolipoproteins concentrations of up to 2.00 µg/mL gave > 85 % cell viability, apoA1, apoB and apoE were considered to be non-toxic towards the Huh7 cell. Therefore, the highest concentration up to 2.00 µg/mL was used for viral internalization study.

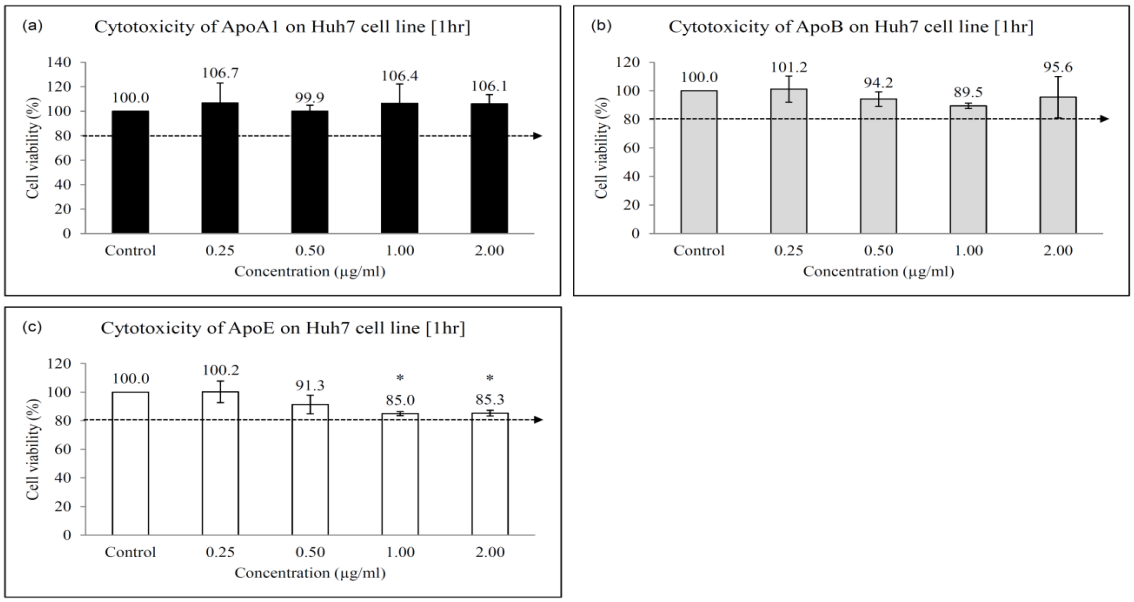

Figure 1 Percentage (%) of Huh7 viability after treatment with various concentrations of apolipoproteins (0.25 - 2.00 µg/mL); (a) apoA1, (b) apoB, and (c) apoE. Data are expressed as means ± SD. Using ANOVA, post-hoc with Bonferroni correction; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to non-treated Huh7 (control).

Effects of apolipoproteins on virus attachment and internalization

Virus binding and internalization assay was adapted from Li *et al.* [17]; Berry and Tse [23] with minor modifications. Both cell surface and internalized virus were measured. In this study, we demonstrated that apolipoproteins increased DENV-2 viral load compared to the non-treated control. apoB exhibited a significant and highest increment compared to apoA1 and apoE ($p = 0.022$ vs $p = 0.323$ and $p = 0.878$, respectively). To validate the finding, we measure the viral internalization indirectly by allowing the bound and internalized virus to grow and replicate for 72 h and viruses released from the host cells in supernatant were collected and measured via qPCR analysis. Theoretically, increase in virus binding and internalization will increase the production of new viruses. Previous *in-vitro* studies reported that DENV replication shows increment in viral titer overtime, reaching optimum level around 48 to 72 h post-infection in various cell lines. Therefore, 72 h incubation was selected in order to provide sufficient viral amplification and production in the supernatant for detection by qPCR. Treatment with apoA1, apoB and apoE increased DENV-2 viral load in supernatant compared to the non-treated control (apoA1 = 92.8 %, apoB = 76.9 % and apoE = 38.7 %), where apoA1 and apoB demonstrated the highest and most significant increment of DENV2 internalization compared to apoE ($p = 0.004$ and $p = 0.016$ vs $p = 0.897$).

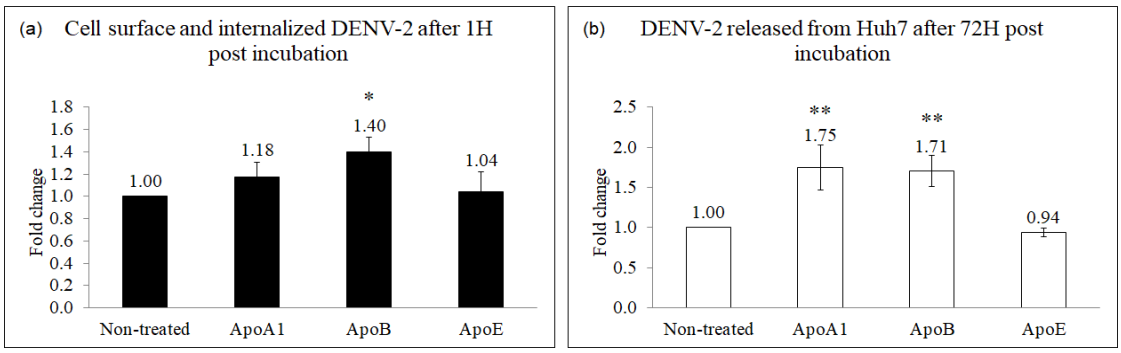

Figure 2 Attachment and internalization of DENV-2 in Huh7. (a) Cell surface and internalized DENV2 after 1 h post incubation and (b) DENV-2 released from Huh7 after 72 h post incubation. Data are expressed as means ± SD. Using ANOVA, post-hoc with Bonferroni correction; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to non-treated Huh7 (control).

The present study revealed that DENV-2 viral load was elevated with the presence of apolipoproteins, particularly apoA1 and apoB. The findings from this study suggests that DENV-2 utilizes both apolipoproteins as transport mediums to migrate closer to the cell surface for viral attachment and internalization. Previous studies have reported that dengue virus nonstructural protein 1 (NS1) having the ability to interact with cellular membranes and liposomes [24-27]. Studies have shown NS1 forming stable complexes by integrating with human HDL-c and LDL-c particles which trigger a pro-inflammatory response [28,29]. Coelho *et al.* [30] reported that NS1-HDL complexes were formed through nonpolar interaction between NS1 protein and apoA1. Benfrid *et al.* [28] described that NS1-HDL complexes structure is in the form of spherical HDL-c particles with rod-shaped NS1 protrusions on their surface and can be detectable in the plasma using anti-apolipoprotein A1 (anti-apoA1) antibodies specific to the HDL-c moiety. They also discovered that the accumulation of these complexes triggers the production of pro-inflammatory cytokines in human primary macrophages while NS1 or HDL-c alone do not [28]. Activated pro-inflammatory cytokines then increase vascular permeability and virion attachment, thus favoring virus replication in infected organisms [28]. Binding determinants for NS1 protein was not exclusive to apoA1 and it can form similar complexes with LDL-c suggesting the involvement of apoB [28]. Therefore, we postulate that DENV-2 may form a complex with free human apoA1 or apoB, leading to alteration of the lipid metabolism for their transmigration to the target organ/cells thus increasing their infectivity. However, a study by Coelho *et al.* [29] reported that apoA1 reduces attachment of dengue virus on cell surface possibly owing to the cells (RAW246.7) used in the experiment which was monocyte from the rats which may have different structure to human monocytes.

siRNA knockdown of SR-B1 receptors

In vitro study on dengue internalization using recombinant apoA1 by Li *et al.* [17] revealed that apoA1 enhanced dengue virus infection via promoting initial attachment of virus to cells through SR-B1 receptor suggesting that respective receptors for apoA1 and apoB might be a potential route of entry for dengue virus infection. In the present study, the SR-B1 was knocked down using siRNA treatment to validate the involvement of both apoA1 and apoB in DENV internalization. The effectiveness of siRNA treatment was measured through qPCR analysis by measuring the gene expression of target receptors. About 50 % knockdown of SR-BI by siRNA in Huh7 was confirmed by mRNA levels based on its GAPDH levels expression after 48 h of siRNA treatment prior to DENV infection and apolipoprotein treatment. Analysis of DENV-2 viral load released from Huh7 by qPCR revealed that down-regulation of SR-B1 by siRNA treatment significantly reduced DENV2 infectivity, verifying that apoA1 does play a crucial role in virus infectivity and is mediated through SR-B1 receptors. **Figure 3** shows a significant reduction of DENV-2 infectivity with SR-B1 down-regulation/silencing (35.2 % reduction, $p = 0.013$). apoB shows no significant difference between non-silent and siRNA-treated cells implying that DENV-2-apoB complex internalization may be mediated by other routes of entry or receptors. While apoE revealed no significant effects compared to non-treated control and between non-silent and siRNA-treated cells.

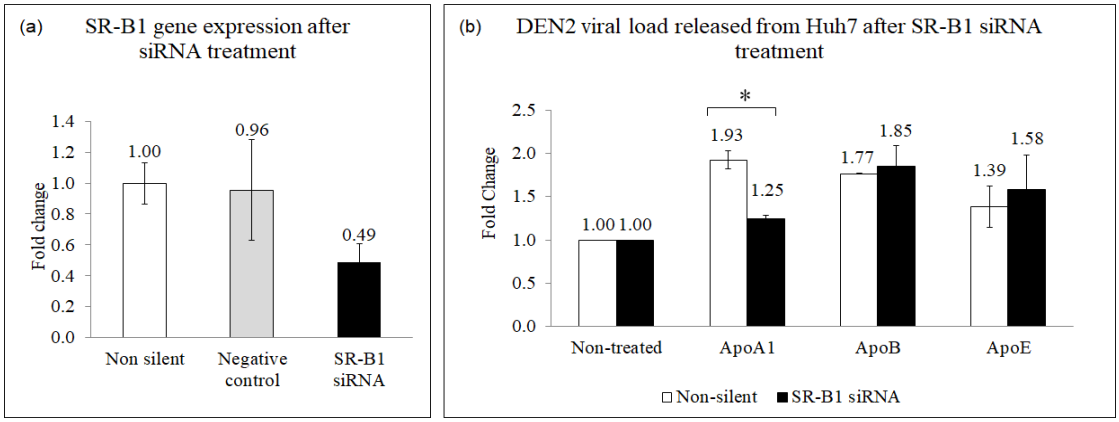

Figure 3 DENV-2 viral load released from Huh7 after SR-B1 siRNA treatment. (a) SR-B1 gene expression after SR-B1 siRNA treatment and (b) Comparison of DENV-2 viral load between non-silent and SR-B1 siRNA treated Huh7. Data are expressed as means ± SD. Using independent T-Test; $^{*}p < 0.05$ compared to non-silent Huh7 (control).

Our data concurred with Li *et al.* [17], where they found that reducing SR-B1 receptors abolished the enhancement of DENV infection by apoA1. Alcalá *et al.* [18] highlighted that SR-B1 in human hepatic cells, and a scavenger receptor B1-like in mosquito C6/36 cells act as cell surface binding receptor for DENV NS1. Since apoA1 is the natural ligand for SR-B1 receptor, this finding proves that the NS1 protein may use apoA1 as a mediator to attach to the cell surface and the same may happen to apoB. Further study on the effects of apoB and interaction with its receptor may provide insight into the functional importance of apolipoproteins in DENV pathogenesis.

Conclusions

In conclusion, the free human apolipoproteins, particularly apoA1 and apoB, in its single form are evident to enhance the initial attachment of DENV-2 to the cell surface, suggesting their role in DENV-2 internalization and infectivity. Our data further revealed that apolipoproteins might have some interaction with DENV-2 viral protein particles to form a complex which allows the transportation of the virus near to cell surface for better infectivity. Moreover, this experiment proves that DENV attachment is aided by the interaction between apoA1 and SR-B1 where the viral load is reduced by the reduction of SR-B1 receptors. However, this situation is different with apoB because the viral load remains high even though SR-B1 has been inhibited indicating that apoB may interact with other receptors.

While our research has contributed valuable insights, there remain areas requiring further exploration. Future studies could delve deeper into the interaction of DENV with other exclusive receptors for apolipoproteins such as Low-Density Lipoprotein Receptor (LDLr) and LDL Receptor-Related Protein (LRP). Additionally, investigating the difference in virus infectivity between DENV serotypes can provide more information since each serotype has its distinct genetic variation.

Overall, this manuscript elucidates the pivotal role of apolipoprotein in DENV internalization, further provides an insight into the mechanism of DENV infectivity and may be used as biomarkers in predicting severity of disease which potentially translate to its utility in dengue risk assessment, stratification and prognostication.

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