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ORIGINAL ARTICLE

The acute effects of nonstructural-1 protein dengue virus type 2 on wet liver weight, zonulin expression and serum zonulin

Safari Wahyu Jatmiko[1,2*], Hartono Hartono[1,3], Tonang Dwi Ardyanto[1,4], Dono Indarto[1,3]

[1]Department of Medical Sciences, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia
[2]Department of Immunology and Molecular Biology, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta, Indonesia
[3]Department of Physiology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia
[4]Department of Clinical Pathology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia

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ABSTRACT

Background and Objectives: Intestinal leakage commonly occurs in severe dengue infection with zonulin as a biomarker. The aim of this study was to determine the effects of NS1 on liver weight, zonulin expression and serum zonulin levels.
Materials and Methods: This laboratory experiment used 18 ddY mice, which were randomly divided into control (C), PBS (T1), and PBS + NS1 (T2) groups. Mice in the T1 and T2 groups were intravenously injected with 500 µl PBS only and 50 µg NS1 respectively. Mice blood samples were collected before and after three-day treatment for measurement of zonulin level. The fresh liver was weighted directly and were then used for immunostaining.
Results: The C group had lower wet liver weight compared to the T groups (p=0.001). Increased expression of liver zonulin was found in the T2 group, significant different from the C (p=0.014) and T1 groups (p=0.020). After treatment, serum zonulin levels in the T1 group was higher than that of the T1 group before treatment (p=0.035) but not in control (p=0.753) and T2 groups (p=0.869).
Conclusion: Administration of 50 µg NS 1 increases wet liver weight and zonulin expression in hepatocytes, but did not increase serum zonulin levels in ddY mice.

Keywords: Acute phase protein; Dengue virus type 2; Hepatomegaly; Nonstructural protein 1; Zonulin

INTRODUCTION

Dengue virus infection (DVI) is the main arthropod-borne virus caused by dengue virus (DENV) types 1-4 and is transmitted through mosquito vectors (1, 2). The worldwide incidence of symptomatic DVI is 58-96 million from which 250,000-500,000 patients had severe DVI. The mortality rates of DVI reached 9,000-24,000 people per year (3). In Indonesia, DVI cases reached 59,047 with 0.75% of mortality rate (4).

The mortality rate in severe DVI is linked to vascular leakage and bleeding (5). The severe DVI pathogenesis has not been explained satisfactorily but it may involve viral proteins (6). The Dengue virus has three structural proteins (capsid, membrane, and

*Corresponding author: Safari Wahyu Jatmiko, MD, MSc, Department of Medical Sciences, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia; Department of Immunology and Molecular Biology, Faculty of Medicine, Universitas Muhammadiyah Surakarta, Surakarta, Indonesia. Tel: +62-2717890444 Fax: +62-2716347400 Email: safari@student.uns.ac.id

envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (7). Non-structural proteins have canonical and non-canonical functions, which are responsible for viral replication, pathogenesis, escape from the immune system, changing in host cell metabolism, and triggering host protein migration (8). Specifically, NS1 can be released into the circulation and which act as a viral toxin (9). Circulating NS1 will be captured by heparan sulfate and chondroitin sulfate E molecules on cell membrane of the hepatocytes and facilitates NS1 concentration within the hepatocytes (10, 11). However, it has not established whether or not the NS1 stimulates the formation of acute phase proteins such as C-reactive protein (CRP), complements, serum amyloid A, pentraxins, and zonulin (Zonulin family protein) (12, 13).

Zonulin (Zonulin family protein) is an acute phase protein, which is encoded by the haptoglobin gene and composes of 406 amino acids with a 45.2 kD molecular weight. Zonulin is highly expressed in the liver and the small intestines, which is secreted in the blood circulation (14). Physiologically, Zonulin expression increases intestinal permeability by shortening the cell cytoskeleton (15). Zona occludens protein 1 (ZO-1) is another protein that plays an important role in linking tight junction transmembrane proteins and the cytoskeleton (16). Therefore, the shortening of cytoskeleton will push in ZO-1 towards the cytoplasm, resulting in opening the gap between enterocytes (15). It causes microbial translocation which triggers inflammation as seen in sepsis and COVID-19 (17, 18). Several studies reported that high intestinal permeability was also found in patients with severe DVI (19, 20).

The role of NS1 increasing zonulin expression has not been investigated. Therefore, the aim of this study was to investigate the effect of NS1 on liver weight, liver zonulin expression, and blood zonulin levels.

MATERIALS AND METHODS

Study design and animals. A laboratory experiment was used in this study with pre-posttests control group design, which was used ddY mice from Integrated Research and Testing Laboratory (Laboratorium Penelitian dan Pengujian Terpadu) Universitas Gadjah Mada Indonesia and conducted at the same place. ddY mice are immunocompetent mice used in dengue research (21). In addition, the Th cells polarization of ddY mice tends towards Th1 cells which is similar to the immune response to viruses (21, 22). The research protocol received ethical approval from the Dr. Moewardi Hospital Health Research Ethics Commission with Number: 435/IV/HREC/2021. Animal number included in this study were 18 according resource equation approach sample size calculation (23). The mice were included in this study with criteria: healthy male, 8-10 weeks old, and weighed 20-30 g. The 18 male mice were randomly divided into 3 groups: control (C) and treatment groups (T1 and T2). The treatment groups were intravenously injected with 500 µl PBS (Sigma Life Science, USA) only and 500 µl PBS (Sigma Life Science, USA) + 50 µg/ml recombinant NS1 DENV2 (R&D, USA) respectively. Interventions were done a day after pretest blood collection.

Measurement of serum zonulin levels. 750 µl venous blood of all mice were taken from orbital plexus, were used for measurement of mice serum zonulin level. The blood samples were collected in day one and four during this study and were further processed to obtain mice serum. Mice zonulin levels were determined using an ELISA kit (abbexa, UK) according to the manufacturer's instructions.

Liver tissue preparation. Liver organs were taken 72 hours after treatment with necropsy technique for mice according Scudamore et al. (24). The liver's wet weight was measured using a Mettler Toledo digital scale (ME802E, Switzerland).

Immunohistochemical (IHC) staining of liver zonulin. The preparation of IHC liver zonulin is briefly described as follows: Paraffin blocks were cut with a thickness of 5 µm. After fixation on to the object glass, deparaffination was carried out and slides were incubated using an anti zonulin primary antibody (Thermo Fischer, USA) according to the manufacturer's instructions and using a secondary IgG antibody Starr Trek Universal HRP Detection System (Starr trek, Biocare Medical, USA), according to the manufacturer's instructions. The slides were then stained with 3,3'-diaminobenzidine (Starr trek, Biocare Medical, USA) and counter stained with Hematoxylin and eosin staining (Merck, Germany). IHC readings use histoscore.

Statistical analysis. Data of wet liver weigh and serum zonulin levels of T1, and T2 groups on day 1 and 4 are presented in mean ± SD. Serum zonulin level of C group on day 4 is presented in mean ± SD, while liver zonulin expression and group C serum zonulin levels on day 1 data are presented in the median (minimum-maximum). All data were analyzed using a Jeffreys's Amazing Statistics Program version 0.16.0.0 free software (University of Amsterdam). The mean differences of serum zonulin level of T1 and T2 groups were examined using the paired t test while wet liver weight was analyzed using the One-way ANOVA which followed by the Tuckey post hoc test. The serum zonulin level of C group was analyzed using the Wilcoxon signed rank, while liver zonulin expression data were examined using Kruskal-Wallis and followed the Dunn's post hoc test with p value <0.05. Tukey's and Dunn's methods are used to avoid type I errors.

RESULTS

Exposure to the NS1 triggers acute phase protein syntheses including zonulin, resulting in changes in liver weight. Based on Table 1 data, we observed that there was a difference in wet liver weight between C and T groups (p=0.001). A greater weight of wet liver was observed in the T2 group (2.32 ± 0.09 g), compared to T1 (1.82 ± 0.22 g) and C (1.21 ± 0.28 g) and it reached significant differences (p=0.008; p<0.001).

Zonulin, an acute phase protein, was more expressed in the liver, as a result of the viral toxin NS1. The result of IHC examination (Fig. 1) showed positive expression of hepatocyte zonulin among mice groups. The T2 group had greater expression of hepatocyte zonulin than the other groups.

Table 1 showed that the T2 group had the highest expression of liver zonulin with histoscore 6 (2-9), compared to T1 and C which had histoscore 3 (2-3) and 3 (1-3) respectively. The NS1 injection increased significantly histoscore of hepatic zonulin (p=0.048) in the day 3. Dunn's post hoc test showed that hepatic zonulin expression in the T2 group was higher than the C group (p= 0.014) and T1 (p=0.020). The histoscores of the C and T1 groups were not different (p=0.445). The comparison of zonulin serum levels on the 1st day with the 4th day of each control and treatment group in mice induced or not by NS1 are presented in Table 2.

Table 2 showed serum zonulin levels among mice groups before and after NS1 treatment. In the day 1 treatment, the average of serum zonulin level in the T2 group (5004.59 ± 5040.11) was higher than that of the C (3444.28 (1056.29-8728.6) and T1 (1714.7 ± 469.69) groups. After NS1 treatment, increased serum zonulin was observed in the T1 group (4031.56 ± 1703.6) and significantly differed from the serum zonulin before treatment (p=0.035). In contrast, the average of serum zonulin level in the T2 group (4631.99 ± 2027.41) reduced but it was not significant different (p=0.869). In addition, the serum zonulin levels in the C group remained stable (3347.02 ± 3627.32). This result is interesting because the T2 group mice were injected with a solution containing PBS plus the recombinant NS1 and there was an increase in zonulin expression in the liver.

DISCUSSION

In this study, we have firstly demonstrated that administration of 50 µg NS1 significantly increased liver weight, liver zonulin expression, but did not increase serum zonulin levels in ddY mice, compared to control and PBS treatment groups. Our finding indicated that NS1 administration increased wet liver weight due to higher expression of acute phase proteins. Exposure to DENV antigens to Kupffer cells increases proinflammatory cytokines (IL-1, IL-6, and TNFα), which induce hepatocytes to produce acute phase proteins such as Alpha-1-antitrypsin precursor, C-Reactive Protein, complements, serum am-

Table 1. Wet liver weigh and liver zonulin histoscore of NS1 treated mice.

	C	T1	T2	p
Wet liver weigh (g)[1]	1.21 ± 0.28	1.82 ± 0.22	2.32 ± 0.09	<0.001*
Liver zonulin expression[2]	3 (1-4)	3 (2-4)	6 (2-9)	0.048*

[1]Wet liver weigh was analyzed using One way ANOVA with Tukey HSD post hoc, [2]Liver zonulin expression was analyzed using Kruskall-Wallis with Dunn post hoc. *Statistically significant.

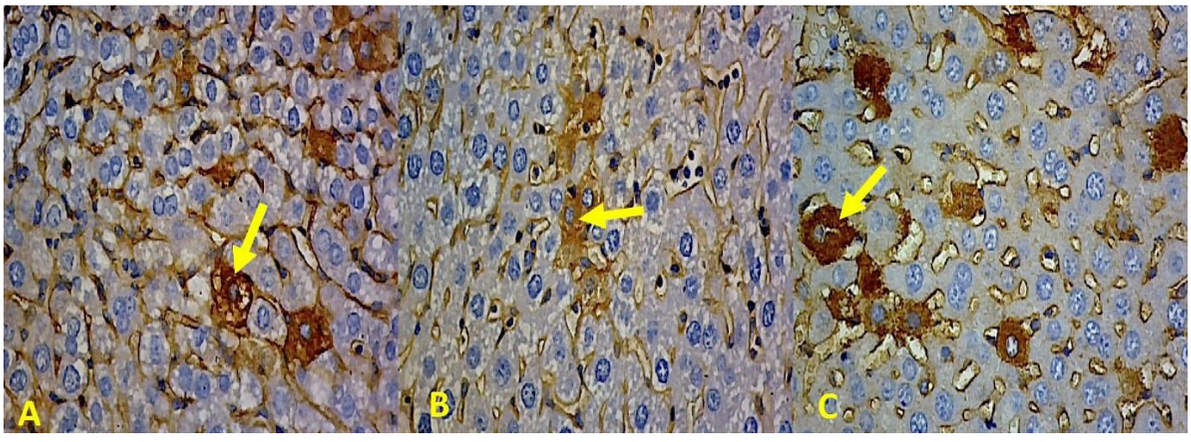

Fig. 1. Immunohistochemical staining of zonulin expression on mice liver treated with NS1.
Five µm thickness of liver dissection were stained using anti zonulin antibody, and were observed using light microscope with 400× magnification. Immunohistochemical showed positive expression of zonulin in hepatocyte cytoplasm (yellow arrows). The histoscore was calculated based on the percentage of cells expressing zonulin and color intensity. One picture represents one group: A. Histoscore = 3 (1-4), B. Histoscore = 3 (2-4), and C. Histoscore = 6 (2-9) with p=0.048.

Table 2. Serum zonulin levels of mice treated with NS1.

	Day		p
	1	4	
Serum zonulin (ng/ml)			
C[1]	3444.28 (1056.29-8728.6)	3347.02 ± 3627.32	0.753
T1[2]	1714.7 ± 469.69	4031.56 ± 1703.6	0.035*
T2[2]	5004.59 ± 5040.11	4631.99 ± 2027.41	0.869

[1]Wilcoxon Signed Rank Test, [2]Paired t test. *Statistically significant.

yloid A (SAA), pentraxin, and zonulin family peptide (12, 13). Specifically, zonulin family peptide and SAA were significantly higher in DVI patients (12). In our study, increased wet liver weight is in line with the result of IHC staining in the hepatocytes, which showed an increase zonulin expression. Another possibility of increased wet liver weight in DENV patients is the vascular leakage in the liver circulation due to not only NS1 but also DENV (25-27). Previous studies showed that administration of sNS1 on day 6 in ADE mice model infected with DENV2 increased vascular permeability in the liver and kidneys (28). The NS1 stimulates formation of sialidase, cathepsin L, heparinase, and hyaluronidase of endothelial cells in the liver, activation of complements, degradation of VE Cadherin, inhibition of hyaluronan-CD44 binding complexes, and reduction of CD44 expression (29-36). The high expression of sialidase, cathepsin L dan heparinase leads to degradation and loss of negative charge of Endothelial-Glycocalyx Layer, resulting in extravasation of albumin into the extravascular compartment (33). However, we were unable to confirm the vascular leakage in the liver of our study. Overall, increased liver weight suggests increased acute phase protein synthesis by hepatocytes and possibly vascular leakage.

In the clinical setting, 70.2% patients with DVI have a clinical manifestation of liver enlargement, which is more prominent in children than in adults (37, 38). From a narrative review, most publications reported that the liver enlargement occurs in response to the DENV. DENV-infected hepatocytes and Kupffer cells lead to apoptosis and necrosis, which induce liver congestion due to vascular leakage and bleeding. However cellular and molecular pathogenesis of these conditions are complicated (27). This was ruled out in this study due to NS1 injection without infection in hepatocytes or Kupffer cells.

So far, the mechanism of intravenous injection of NS1 increasing zonulin expression in the liver is un-

known. Several studies indicated that NS1 increase the syntheses of proinflammation cytokines such as IL-1, TNF-α, IL-6, and IL-18 which induce the production of acute phase protein such as zonulin (12, 39-42). Increased proinflammatory cytokines are known to occur in other viral infections such as COVID-19 (43). In contrast to the NS1 administration, viral hepatitis B and C viral infections cause hepatocyte damage, leading to reduce zonulin synthesis (44). Therefore, our finding suggests that administration of NS1 results in the increase of proinflammation cytokines and acute phase proteins including zonulin.

Our results showed that after 3 days intervention, serum zonulin levels in the T1 group increased higher than before intervention while serum zonulin levels in the T2 groups remain stable before and after intervention. The increased serum zonulin level in mice without NS1 induction (T1 groups) is unknown, but there are previous studies showing that PBS increases the formation of proinflammatory cytokines of neutrophils and monocytes (45, 46). In addition, administration of PBS only increased synthesis of acute phase proteins such as SAA, fibrinogen, CXCL-1, and zonulin in mice treated with and without IL-22 intraperitoneal injection (47, 48). In contrast to the result in the T1 group, serum zonulin level decreased slightly in the T2 mice group. Based on computational studies, NS1 may interact with zonulin to generate zonulin/NS1 complexes (35, 49). We think that the decrease in zonulin on T2 is due to the formation NS1/zonulin complexes. This complex may not be recognized by the anti-zonulin antibody used in this study.

There were some limitations from our study, which probably influence the wet weight liver and serum zonulin level. We did not explore the effect of proinflammatory cytokines on zonulin formation in the other mice cells like small intestine cells, immunocytes, and lung cells. Secondly, we did not identify other acute phase proteins after the NS1 administration, which also contribute to increase wet weight liver and hepatic vascular leakage. Thirdly, we did not examine the presence of NS1/zonulin complexes that may occur post zonulin secretion due to NS1.

CONCLUSION

Administration of 50 µg NS1 increases wet liver weight and zonulin expression in the liver cells, but did not increase serum zonulin levels in ddY mice. It is necessary to investigate further the roles of proinflammatory cytokines and acute phase proteins other than zonulin in promoting hepatic vascular leakage. In addition, further research is needed to determine the possibility of the formation of the NS1/zonulin complex and its effect on the pathogenesis of DVI.

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