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The Intestinal Zonulin and Zonula Occludens 1 Protein Expression and Lipopolysaccharide Levels In ddY Mice Injected with Dengue Virus Non-Structural Protein 1

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ABSTRACT

Background and Aim: Dengue virus infection remains a health problem. Dengue Virus Non-Structural protein 1 (NS1) increases the release of proinflammatory cytokines that induce intestinal zonulin expression. As a result, the ZO-1 protein translocates to the cytoplasm, which increases enterocyte permeability. This study aimed to investigate the effects of dengue NS1 on intestinal zonulin and ZO-1 expression, intestinal weight, and serum LPS.

Materials and Methods: This experiment used 18 ddY mice with a pre-posttest control group design. Mice were randomly divided into control (C), PBS (T1), and PBS+NS1 (T2) groups. Mice in the T1 and 2 groups were intravenously injected with 500 µL PBS and 50 µg NS1, respectively. Blood samples were collected before and after the three-day treatment. The intestines were weighted directly and were then embedded with formalin for immunostaining. Serum LPS were determined using ELISA. Data were analyzed using paired t-test and ANOVA.

Results: The T2 group had the highest zonulin expression (histoscore=8) compared to the T1 (histoscores=7.33) and C (histoscore=6.33) groups but were not significant (*P*=0.135). Intestinal ZO-1 expression did not increase in the T groups compared to the C group (*P*=0.368). The intestine weight in the C group was significantly lower than the T group (*P*=0.001). After treatment, serum LPS levels in the T2 group were higher (0.34 pg/mL) than before treatment (0.12 pg/mL; *P*=0.118), but not in the T1 group (*P*=0.384). Meanwhile, there was a significant decrease in serum LPS levels in the C group (*P*=0.046).

Conclusion: injection of 50 µg dengue virus NS1 has a minor effect on intestinal permeability in ddY mice.

Keywords: Dengue virus, LPS, NS1, Zonulin, Zonula occludens 1

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1. Introduction

Dengue virus infection (DVI), which is caused by the dengue virus (DENV), remains a health problem in tropical and subtropical countries, including Indonesia **(1, 2)**. The DVI incidence reaches 58-96 million worldwide **(3)** and increases with urbanization **(4)**, global warming **(5)**, and tourism **(6)**. The DVI mortality reaches 9,000 – 24,000 people each year **(3)**. The definitive treatment and vaccination for DVI have not been established because the mutation rate of DENV genome structure remains high, and the DVI pathogenesis remains unclear **(7-9)**. However, the viral structure may be involved in DVI pathogenesis **(10)**.

The DENV genome has three structural proteins (capsid, membrane, and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) **(10)**. The NS2A and B protein plays vital roles in RNA synthesis and in breaking down the transcriptional protein chain into structural and non-structural proteins with the NS3 protein **(11, 12)**. Meanwhile, the NS4A and B proteins regulate replication **(13)** and interact with other viral proteins for replication **(14)**. The NS5 and NS1 proteins function as an RNA-dependent RNA polymerase **(15)** and a trigger factor for severe DVI, respectively **(16)**. In addition, the NS1 can activate immunocytes such as monocytes, B cells, and dendritic cells through Toll-like receptor (TLR) activation, mainly TLR-4 **(17, 18)**, TLR-2, and 6 **(19)**, resulting in the release of proinflammatory cytokines **(20)**.

A previous study reported that increased blood Lipopolysaccharide (LPS) levels are detected in patients with severe DVI, but the mechanism is not fully understood **(21)**. Theoretically, the LPS is the outer wall structure of gram-negative bacteria, composed of lipid A, oligosaccharide core, and O antigen chain **(22)**. The increased blood LPS levels may occur due to damage of the intestinal epithelium **(23)** or related to immune activation **(24)**, which induces the synthesis and secretion of proinflammatory cytokines through interaction with the TLR4 **(25)**.

Dengue virus can infect keratinocytes, monocytes, dendritic cells, and macrophages **(26)**. Dengue virus replication generates new virions and viral particles such as NS-1 **(10)**. Non-structural 1 activates immunocytes to produce proinflammatory cytokines such as interleukin 1 (IL-1), Tumor necrosis factor-α (TNF-α), and IL-6 through TLR-4 activation **(18, 20, 27)**. These cytokines enter the blood circulation and affect various body organs, including the gut. It has been reported that those proinflammatory cytokines promote intestinal zonulin expression **(28)**.

Zonulin is a structural protein with a 42 kDa molecule weight, which functionally regulates cell interconnection and permeability for ion, water, and nutrient transports **(29, 30)**. This protein is highly expressed in the small intestine and liver **(31)** and has a high homology with the zonula toxin of *Vibrio cholerae* **(30)**. Moreover, high expression of zonulin triggers the translocation of zonula occluden-1 (ZO-1) from the outer cytoplasm into the inner cytoplasm of enterocytes **(32)**.

The zonula occluden-1 is a family member of membrane-associated guanylate kinase)-like proteins with a 220 kDa molecular weight. This protein interacts with other structural proteins to form the intestinal tight junction (TJ) between the cell membrane and the cytoskeleton **(33)**. During inflammation with various causes, increased TNF-α and IL-6 levels can cause high expression of the intestinal zonulin, leading to translocation of ZO-1 to open the gap of enterocyte connection and followed by microbial translocation, including gram-negative bacteria **(34, 35)**. For example, severe acute respiratory syndrome of coronavirus 2 infection increases zonulin expression leading to intestinal leakage **(36, 37)**. However, limited studies investigated the role of DENV NS1 on intestinal zonulin expression, which was linked to intestinal leakage in response to cytokine storms. Therefore, this study aimed to investigate the effects of DENV NS1 injection on intestinal zonulin and ZO-1 expression, wet intestinal weight, and serum LPS level in ddY mice. Specifically, we would evaluate the roles of intestinal zonulin and ZO-1 expression and serum LPS level as potential markers for intestinal leakage. Intestinal leakage has been reported in patients with DVI **(21)** in which the NS1 protein is probably involved in intestinal leakage. Therefore, the novelty of our study is that the NS1 protein might induce intestinal leakage by changing the expression of zonulin and ZO-1.

2. Materials and Methods

This laboratory experiment used ddY mice with a pre-posttest control group design for LPS measurement and a posttest-only control group design for wet intestinal weight, zonulin, and ZO-1 expression. This research study was part of a big research project related to DVI pathogenesis. The Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada, Yogyakarta, Indonesia, provided these mice, and the research study was conducted at the same place. The sample size was calculated using the resource equation approach with the formula n = DF/k + 1 (DF= degrees of freedom, K= number of groups) to obtain 18 mice for all groups in this experiment **(38)**. The mice selection used the inclusion criteria: healthy male, 8-10 weeks old, and weighing 20-30 g. Selected mice were randomly divided into control (C) and treatment (T1 and T2) groups. The mice were acclimatized with the following conditions: each group was housed in a plastic cage, sawdust bedding, 25^{o}C temperature, > 50% humidity, 12/12 hours cycle of dark and light, and keeping away from electronic equipment and noise. All mice were given a grower animal feed (JAPFA Comfeed Indonesia, Comfeed AD II) with 10% body weight (bw) per day and drinking water ad libitum. The T1-2 groups were intravenously injected with a single dose of 500 µL PBS (Sigma Life Science, USA) only and 500 µL PBS (Sigma Life Science, USA) + 50 µg NS1 DENV2 (R&D, USA), respectively.

Intestinal Weight Measurement

In the day 3 treatment, all mice were sacrificed and the intestine was taken using the necropsy technique, according to Scudamore, Busk, *et al.* **(39)**. The intestine was directly weighted using a Mettler Toledo digital scale (ME802E, Switzerland).

Immunohistochemical (IHC) Staining of Intestinal Zonulin and Zonula Occludens 1

The immunostaining method in this research study used a standard method routinely conducted in the Anatomical Pathology Laboratory, Faculty of Medicine Universitas Sebelas Maret (UNS). After measurement of wet intestinal weight, the mice's small intestines were embedded using paraffin and cut into 5 µm thickness. After fixation on the object glass, deparaffination was carried out and slides were incubated using an anti-zonulin primary antibody (Thermo Fischer, USA) for zonulin staining, while the ZO-1 staining used anti ZO-1 antibody (Thermo Fischer, USA), 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). The Histoscore for positive cells expressing zonulin and ZO-1 was calculated by multiplication of the percentage of stained cells and the color intensity. The scoring of stained cells was assessed in the following manner: 0, 1, 2, 3, and 4 scores for 0%; 1-25%; 26-50%; 51-75%; and 76-100% respectively. The color intensity was assessed 0 score: non stained, 1 score: stained with low intensity, 2 score: stained with moderate intensity, and 3 score stained with strong intensity.

Measurement of Serum Lipopolysaccharide Levels

Before and after treatment, 750 µL venous blood samples of all mice were taken from the orbital plexus, which were further processed to obtain mice serum. Mice serum LPS levels were determined using an ELISA kit (Abbexa, UK) according to the manufacturer's instructions.

Statistical Analysis

All collected data, such as wet intestinal weight, intestinal zonulin and ZO-1 expression, and serum LPS levels, were presented as mean ± SD and were analyzed using a JASP 0.16.0.0 free software (University of Amsterdam). The mean differences in LPS serum levels of the T1 and T2 groups were examined using the paired t-test, while intestinal weight was analyzed using the One-way ANOVA, followed by the Tuckey post hoc test. LPS serum data were examined using the Wilcoxon signed-rank tests. The Kruskal-Wallis was used to compare the mean intestinal zonulin and ZO-1 among groups and followed Dunn's post hoc test with a p-value <0.05. For further analysis of statistical significance, the effect size and Bayes factor 10 (BF10) of all collected data were used **(18)**. BF10 value > 1 indicates significance for all statistical analyses. The ω2 > 0.01 was used to determine an effect size for the one-way ANOVA, Cohen's d > 0.2 for the paired t-test, and rB > 0.1 for the Wilcoxon signed-rank test.

3. Results

Table 1 indicated that administration of 50 µg DENV NS1 slightly increased intestinal zonulin expression but did not affect intestinal ZO-1 expression. In addition, NS1 did not increase wet intestinal weight. The histoscore of intestinal zonulin expression increased, although not statistically significant (*P*=0.135). Intestinal ZO-1 expression remained unchanged among groups. The mean intestinal weight in the T2 group was significantly higher than that of the C group (*P*<0.001, d=2.659, and BF_{10}= 25.8), but there was no difference between the T2 and T1 group (*P*=0.482, d=0.482, and BF_{10}=0.685).

Table 1. The comparison of intestinal weight, zonulin, and ZO-1 expression on mice treated with and without NS1 Ag.

Variable	C	T1	T2	p
Intestinal Zonulin Expression[1]	6.33 ± 1.97 [a(NS)]	7.33 ± 1.633 [a(NS)]	8 ± 0.00 [a(NS)]	0.135
Intestinal ZO-1 Expression[1]	8 ± 0.00 [a(NS)]	7.33 ± 1.63 [a(NS)]	8 ± 0.00 [a(NS)]	0.368
Intestinal weight (g)[2]	3,87 ± 0.58 [a]	5,19 ± 0.64 [b(NS)]	5.64 ± 0.77[b(NS)]	0.001*

All data were presented as mean±SD. The Histoscore for positive cells expressing zonulin and ZO-1 was calculated by multiplication of the percentage of stained cells and the color intensity.

[1] analyzed using the Kruskal-Wallis test and followed by Dunn's post hoc with *P*>0.05, [a] designated the comparison of C-T1, C-T2, and T1-T2.

[2] Analyzed using the One-way ANOVA test and followed by post hoc Tukey's with *P*<0.05. [a] designated comparison of C-T1 and C-T2. [b] designated the comparison of T1-T2. * Statistically significant, BF_{10} = 34.2, ω2 = 0,537.

Figure 1 showed that the intestinal zonulin expression was similar among groups (*P*=0.135). However, higher expression of zonulin was observed in the T2 group (histoscore = 8 ± 0.00) compared to the T1 (histoscore = 7.33 ± 1.63) and C (histoscore = 6.33 ± 1.97) groups.

In terms of ZO-1 expression, it had similar histoscores to the zonulin expression. Figure 2 showed that positive immunostaining of ZO-1 was similar among groups, and the histoscores did not differ significantly (*P*=0.368). The ZO-1 expression in the T2 group (histoscore=8 ± 0.00) was similar to the ZO-1 expression in the C group (histoscore=8±0.00). Meanwhile, the T1 group had lower ZO-1 expression (histoscore=7.33±1.63).

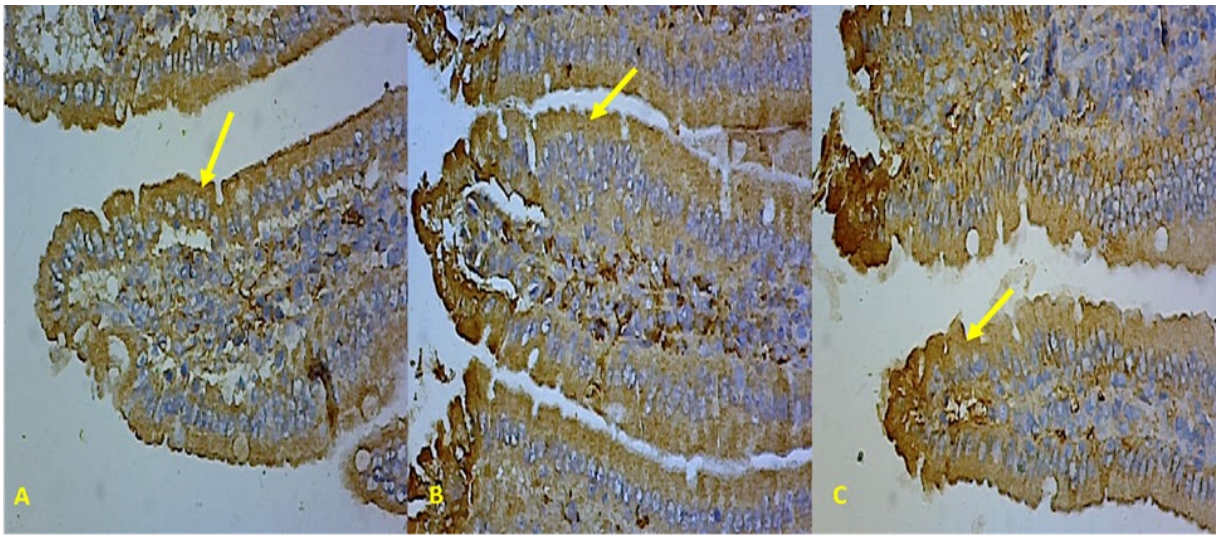

Figure 1. Immunohistochemical staining of zonulin expression on mice intestine treated with and without NS1 Ag. Five µm thickness of intestine dissection was stained using an anti-zonulin antibody and was observed using a light microscope with 400x magnification. Yellow arrows designated positive immunostaining of zonulin in enterocytes. The histoscore of positive immunostaining was based on the percentage of cells expressing zonulin and color intensity. Each picture represented six mice/group and had an A. Histoscore 6.33 ± 1.97, B. Histoscore 7.33 ± 1.633, and C. Histoscore 8 ± 0.00 with *P*=0.135.

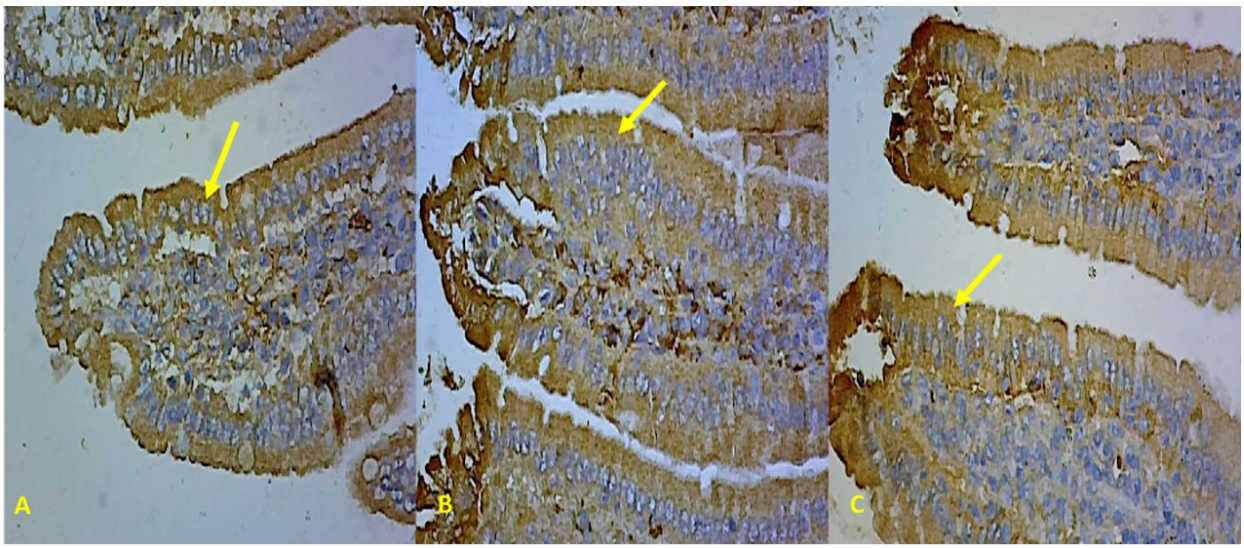

Figure 2. Immunohistochemical staining of ZO-1 expression on mice intestine treated with and without NS1 Ag. Five µm thickness of intestine dissection was stained using an antiZO-1 antibody and was observed using a light microscope with 400x magnification. Yellow arrows designated positive immunostaining of ZO-1 in enterocytes. The histoscore of positive immunostaining was based on the percentage of cells expressing ZO-1 and color intensity. Each picture represented six mice/group and had an A. Histoscore 8 ± 0.00, B. Histoscore 7.33 ± 1.63, and C. Histoscore 8 ± 0.00 with *P*= 0.368.

Tables 2. Changes of LPS serum in mice treated with and without NS-1

Variable	Day		*P*
	1	4	
LPS (pg/mL)			
C[2]	0.66 ± 0.48	0.19 ± 0.1	0.046*
T1[2]	0.36 ± 0.43	0.69 ± 0.51	0.384**
T2[1]	0.12 ± 0.1	0.34 ± 0.3	0.118***

[1]Wilcoxon Signed Rank Test, [2]paired t test. * Statistically significant with d=1.078, and BF_{10}=2.221, ** d= 0.39 but it was not statistically significant with BF_{10}=0.53, *** BF_{10}=1.383 and rB=0.714 but statistically not significant.

We examined LPS serum to confirm whether NS1 treatment or not increased intestinal permeability. Table 2 indicated that NS-1 treatment increased intestinal permeability in the T2 group. LPS Serum levels in the T2 group significantly increased by 283% with BF10=1.383 and rB=0.714, but it was not statistically significant with *P*=0.118. The serum LPS levels in the T1 group significantly increased by 191% with d= 0.39, but it was not statistically significant with *P*=0.384 and BF10=0.53. Meanwhile, serum LPS levels in the C group decreased significantly (*P*=0.046, d=1.078, and BF10=2.221).

4. Discussion

Herein, we demonstrated that administration of 50 µg NS1 and PBS slightly increased the expression of intestinal zonulin, but the expression of ZO-1 remained unchanged in ddY mice. In addition, a similar result was found in the wet intestinal weight of ddY mice treated with the same dose of NS1. Meanwhile, LPS serum levels in ddY mice treated with NS1 were higher than in the control and PBS administration groups. Therefore, it suggests that NS1 injection has a minor effect on the permeability of the small intestine.

Zonulin is a tight junction regulatory protein, which is highly expressed in the small intestine induced by various factors such as pathogens, intestinal bacteria, foods, and proinflammatory cytokines **(31, 32)**. Previous studies reported that exposure of gram-negative bacteria to the Wistar rats, New Zealand White rabbits, and Kunming mice induced zonulin expression in the enterocytes **(40, 41)**. In contrast to those findings, our results showed that the expression of intestinal zonulin slightly increased in ddY mice treated with NS1. Our finding is in accordance with a previous study that 10 mg/kg of intravenous NS1 injection in AG129 mice did not cause epithelial damage of the small intestine. Still, it accumulated in the hepatocytes **(42)**. However, the author did not assess zonulin expression in the hepatocytes. The possible mechanism is that the NS1 has a higher binding affinity to the endothelial surface of the liver and the pulmonary blood vessels than that of the intestinal and brain endothelial cells **(43)**.

The ZO-1 is another structural protein that becomes a part of the TJ proteins to connect to the epithelial and endothelial cytoskeletons **(44)**. Our study showed that intestinal ZO-1 expression in all mice groups remained stable. We did not find any similar research study that evaluated ZO-1 expression in mice treated with NS1. Our findings differ from an existing study that orally administering transmissible gastroenteritis virus to DLY-weaned piglets decreased intestinal ZO-1 expression **(45)**. The decreased ZO-1 expression is also detected in mice and rats treated with dextran sodium sulfate **(46)**, trinitrobenzene sulfonic acid **(47)**, and dinitrobenzene sulfonic acid **(48)**. Although NS1 did not affect changes in ZO-1 expression in our study, several reports indicate that NS1 affects the expression of various substances. Intravenous and intraperitoneal injections of 50 µg NS1 into BALB/c mice increased the expression of endothelial macrophage migration inhibitory factor and MMP-9 **(49, 50)**. Furthermore, ddY mice were injected with 50 µg NS1 intravenously, increasing hepatocyte zonulin expression **(51)**. Altogether, it suggests that the NS1 influences zonulin expression in a tissue-specific manner, and perhaps the small intestine is not a specific target of the NS1.

A macroscopic examination of the small intestine is required to assess the NS-1 effect during DVI. Our finding indicated no difference in wet intestinal weight in ddY mice treated with PBS or NS1, although the wet intestine weight of both groups was heavier than the control group. Our results differ from previous research in that the small intestine was swollen in mice inoculated with dengue-antibodies immune complexes **(52)**. The presence of PBS might cause an increase in wet intestinal weight in our study due to inhibitions of carboxylase activity and nutrient absorption. The phosphate ion inhibits the activity of the carboxypeptidase in the brush border of intestinal and pancreatic epithelial cells, which reduces the degradation and absorption of partially digested proteins **(53)**. The undigested proteins, therefore, accumulate in the lumen and mucosal layer of the small intestine, leading to an increase in wet intestinal weight. Another possible reason is that NS1 intravenous injection was predominantly found in the liver of mice, and line with the study performed by Alcon-LePoder and Drouet, *et al.* **(54)**. Furthermore,

the NS1 is more likely to stick tightly to the endothelial surface of the liver and the pulmonary blood vessels than that of the intestinal and brain endothelial cells **(43)**. In contrast, repeated infections of intact DENV may cause more severe disorders in the gastrointestinal tract than primary infection of intact DENV **(52)**.

In this study, we further evaluated serum LPS levels to determine whether the translocation of intestinal microbial and their products from the intestinal lumen to the blood circulation occurred in ddY mice treated with 50 µg NS1. Our results indicated that NS1 administration significantly increased serum LPS levels compared to the ddY mice treated with PBS only and the control groups. To our knowledge, no published article reported the serum LPS level in mice treated with NS1. In the clinical setting, research by van de Weg *et al.* (2013) in Sao Paulo, Brazil, showed that there were significantly increased blood LPS levels in severe DVI patients **(24)**. Our study's results of intestinal zonulin and ZO-1 expression and wet intestinal weight indicate that LPS did not originate from the small intestine. We think the LPS probably comes from the colon lumen for two reasons. Firstly, microbial translocation in severe dengue is associated with the activation of the immune system. Immune activation increases proinflammatory cytokines that trigger increased intestinal permeability **(24)**. Secondly, leukocyte infiltration of the colon submucosa was found in AG129 mice infected with DENV-3 **(55)**. However, we cannot confirm it because we did not examine pathological processes in the colon of mice treated with the NS1. We have some limitations in our study. At first, we did not examine the presence of NS1 in the liver and intestines, which directly influenced changes in zonulin and ZO-1 expression. Secondly, we did not investigate zonulin and ZO-1 expression in the colon and wet colon weight, which may cause epithelial damage and increase serum LPS levels.

5- Conclusion

Intravenous injection of 50 µg DENV NS1 increases serum LPS levels but does not increase zonulin expression, ZO-1 expression, and wet intestinal weight. It suggests that NS1 injection has a minor effect on the permeability of the small intestine. Further research is required to determine the presence of NS1 in the small intestine and the expression of zonulin and ZO-1 in the colon. In addition, we need histopathological staining to evaluate epithelial integrity in the colon.

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Ethics Approval

The research protocol was approved by the Health Research Ethics Commission General Hospital Dr. Moewardi Surakarta, Indonesia, with Number: 435/IV/HREC/2021.

Authors' Contribution

Conception and design of the study: SWJ, H, TDA, DI. Acquisition of data: SWJ. Analysis and/or interpretation of data: SWJ, H, TDA, DI. Drafting the manuscript: SWJ, H, TDA, DI. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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