Jurnal Implementa Husada (JIH)
Vol. 7, No. 4, November 2026
ISSN: 2722-0877

The Role of Dengue Virus Nonstructural Protein 1 (NS1) on Cell Apoptosis through Caspase-3mRNA Expression and Mitochondrial Function

Riyanda Indrawan Sani[1], Nurfadly[2], Zukhrofi Muzar[3], Shahrul Rahman[4], Siti Masliana Siregar[5]

[1] Master of Biomedical Study Program, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia
[2] Department of Parasitology, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia
[3] Department of Histology, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia
[4] Internal Medicine Department, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia
[5] ENT Head and Neck Department, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia

Article Info

Article history:

Received March 10, 2026
Revised September 03, 2026
Accepted September 09, 2026

Keywords:

Apoptosis
Caspase-3
Dengue virus
NS1 protein

ABSTRACT

Background: Dengue virus (DENV) Non-Structural Protein 1 (NS1) contributes to dengue pathogenesis and may affect cellular responses related to mitochondrial function and apoptosis.
Objective: To investigate the effects of recombinant DENV-2 NS1 on mitochondrial membrane potential-related responses and Caspase-3 mRNA expression in human peripheral blood mononuclear cells (PBMCs).
Methods: PBMCs from three healthy donors (n = 3) were exposed to recombinant DENV-2 NS1 at 1 and 5 µg/mL. JC-1 staining was used to assess the proportion of JC-1-positive cells, while Caspase-3 mRNA was analyzed by RT-qPCR. Donors were considered the experimental units.
Results: NS1 exposure did not significantly affect monocyte counts. The proportion of JC-1-positive cells was 0.892 ± 0.024 in controls, 0.686 ± 0.104 at 1 µg/mL, and 0.695 ± 0.031 at 5 µg/mL. Caspase-3 donor-level ΔCt values differed significantly between control and NS1 1 µg/mL groups ($p = 0.007$).
Conclusion: DENV-2 NS1 exposure was associated with changes in JC-1-positive cell proportion and Caspase-3 transcript abundance in human PBMCs. These findings are preliminary and do not establish direct Caspase-3 activation or apoptosis. Further studies using validated reference genes and functional apoptosis assays are warranted.

This is an open access article under the CC BY-SA license.

Corresponding Author:

Riyanda Indrawan Sani
Master of Biomedical Study Program, Faculty of Medicine & Health Sciences, University of Muhammadiyah North Sumatra, Medan, Indonesia
Email: riyandaindrawan@umsu.ac.id

INTRODUCTION

Dengue virus (DENV) infection represents a major global public health challenge, with dengue incidence showing substantial increases over recent decades.[1,2] Dengue is a mosquito-borne viral disease with a broad geographic distribution, with an estimated 390 million DENV infections occurring annually worldwide.[2] Dengue remains an important public health problem in Southeast Asia, including Indonesia, where a seroprevalence of 69.4% was reported in a representative urban pediatric population in 2014.[3,4]

Although most DENV infections are asymptomatic or mild, some patients develop severe dengue characterized by increased vascular permeability and plasma leakage, which can lead to life-threatening complications.[5] Furthermore, neurological manifestations, including encephalopathy, encephalitis, and peripheral neurological complications such as Guillain-Barré syndrome (GBS), have also been reported, indicating that the clinical impact of dengue extends beyond the vascular system.[6]

The DENV genome encodes three structural and seven non-structural proteins, among which non-structural protein 1 (NS1) has attracted considerable attention because of its roles in viral replication and disease pathogenesis.[7] NS1 is a highly conserved glycoprotein that participates in viral replication and contributes to dengue pathogenesis.[8] NS1 contributes to viral RNA replication and is also secreted into the circulation, where circulating NS1 antigenemia has been associated with clinical characteristics and disease severity.[7,8] Secreted NS1 forms a lipid-associated hexamer and can interact with host endothelial components, including the glycocalyx, contributing to complement activation, endothelial dysfunction, and increased vascular permeability.[8,9] NS1 can disrupt endothelial glycocalyx and cell-junction integrity, including tight and adherens junctions, thereby contributing to vascular leakage, a hallmark of severe dengue.[9,10] DENV infection may also involve the central nervous system and the blood–brain barrier, particularly in patients with neurological manifestations.[6,11,12]

Although the vascular effects of NS1 have been extensively investigated, its effects on immune cells, particularly monocytes, remain less well characterized.[13–15] Peripheral blood mononuclear cells (PBMCs), consisting primarily of lymphocytes and monocytes, are important components of the host immune response, with monocytes representing important target cells for DENV infection.[15,16] DENV infection can induce inflammatory responses and apoptosis in monocytes, with differences in cytokine responses observed among monocyte subsets.[15,17,18]

Recent studies have demonstrated that NS1 can interact with lipids and modulate inflammatory responses in monocyte-derived cells.[8,13,14] However, the specific effects of recombinant NS1 on apoptosis-associated responses in primary human PBMCs remain incompletely characterized,[13,14,17] particularly because most previous studies used live whole viruses or immortalized cancer cells, making it difficult to isolate the direct effects of pure NS1, and the results may not fully represent the physiological responses of human immune cells.

One cellular mechanism implicated in DENV pathogenesis involves alterations in mitochondrial function and apoptosis-associated signaling.[17,19–21] Mitochondria play a central role in cellular survival and apoptosis, and disruption of mitochondrial homeostasis can alter mitochondrial membrane potential and apoptosis-associated signaling, including pathways involving executioner caspases such as Caspase-3.[19–21] Changes in mitochondrial membrane potential can provide information regarding mitochondrial functional status and cellular stress.[19,21] In DENV infection, alterations in mitochondrial dynamics and function have been associated with cellular stress and apoptosis, suggesting that mitochondrial disturbances may contribute to cellular injury during infection.[20,21]

Several knowledge gaps underlie the necessity of this study. Previous studies have investigated DENV-associated apoptosis and mitochondrial responses using infectious virus or specific cellular models; however, the direct effects of recombinant NS1 on primary human immune cells remain less well characterized, so the specific effects of pure NS1 on human immune cells have not been systematically isolated and studied.[19] Studies of DENV-associated apoptosis have also used established cell models, which may not fully reproduce the responses of primary human immune cells,[19,20] that may not fully represent the physiological responses of human immune cells to DENV infection; thus, a more relevant model such as primary human PBMCs is

needed. Few studies have simultaneously examined apoptosis-associated Caspase-3 transcript abundance and mitochondrial responses following exposure to recombinant NS1 in primary human PBMCs to demonstrate how NS1 induces an integrated cellular response ranging from transcriptional activation of apoptosis to impairment of mitochondrial function and loss of cellular viability.[19] Although circulating NS1 antigenemia has been associated with clinical disease characteristics and severity, the mechanisms linking NS1 to immune-cell dysfunction and apoptosis remain incompletely understood, so research capable of elucidating these pathways is urgently needed for the development of effective therapeutic strategies.[8,13,22]

In this study, primary human PBMCs were exposed to recombinant DENV-2 NS1 to examine Caspase-3 mRNA expression and mitochondrial membrane potential-related responses to explore the effects of pure recombinant NS1 protein on Caspase-3 mRNA expression and mitochondrial membrane potential, thereby providing a more accurate representation of natural immune cell responses to the viral protein without the confounding effects of whole virus infection. This study adopts an integrative approach by measuring two key parameters simultaneously— Caspase-3 mRNA abundance as an apoptosis-associated molecular response and mitochondrial membrane potential-related changes assessed using JC-1 fluorescence—enabling a more holistic understanding of how NS1 affects cells from the transcriptional level to organelle function. The use of recombinant NS1 allows the cellular response to be examined in the absence of other viral proteins and infectious viral replication, which is may provide a basis for further investigation of the cellular effects of NS1 to inhibit NS1's pathogenic actions without disrupting normal cellular functions. These findings may contribute to further mechanistic investigation of cellular responses induced by DENV NS1 in primary human immune cells. Therefore, this study aimed to investigate the effects of recombinant DENV-2 NS1 on Caspase-3 mRNA expression and mitochondrial membrane potential-related responses in primary human PBMCs, which is expected to provide important contributions to understanding the molecular pathogenesis of dengue and pave the way for the development of more effective diagnostic and therapeutic strategies.

METHOD

This study employed an experimental *in vitro* design to investigate the effects of recombinant Dengue Virus Non-Structural Protein 1 (NS1) on mitochondrial membrane potential-related cellular responses and Caspase-3 mRNA expression in human peripheral blood mononuclear cells (PBMCs). The research was conducted at the Integrated Laboratory of the Faculty of Medicine, Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia, over a period of one year. The study protocol was reviewed and approved by the Health Research Ethics Committee of Universitas Muhammadiyah Sumatera Utara (Ethical Approval Letter No. 1359/KEPK/FKUMSU/2024). All procedures involving human blood samples were performed in accordance with the principles of the 1964 Helsinki Declaration and its later amendments.

Blood samples were collected from three healthy adult donors, representing three independent biological replicates (n = 3). Donors met the following inclusion criteria: age between 20 and 25 years, no history of acute or chronic infectious diseases in the past four weeks, no history of dengue virus infection or vaccination, no autoimmune disorders or immunosuppressive conditions, and no pregnancy or lactation. Exclusion criteria included a positive serological test for dengue at recruitment, acute febrile illness, use of immunosuppressive medications, and a history of allergic reactions to blood collection materials. All donors provided written informed consent prior to participation. The three donors were male, aged 20, 21, and 22 years, respectively, and had normal complete blood count parameters with no reported underlying medical conditions.

PBMCs were isolated from fresh whole blood collected in EDTA-containing tubes using density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, USA). Briefly, 5 mL of whole blood was layered over 5 mL of Histopaque-1077 and centrifuged at 1010 × *g* for 30 minutes at room temperature without brake. The buffy coat layer containing PBMCs was aspirated, transferred to a new tube, and washed twice with phosphate-buffered saline (PBS; Gibco, USA) by centrifugation at 605 × *g* for 10 minutes. The final cell pellet was resuspended in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, USA). Cell viability and total cell count were determined using the trypan blue exclusion method and a Neubauer hemocytometer. To

minimize potential bias and inter-assay variability, all cell culture procedures and subsequent analyses were performed in a randomized and blinded manner within a single experimental batch.

Recombinant dengue virus type 2 NS1 protein (DENV-2 NS1, AAC59275; MedChemExpress, USA) was used in this study. The protein was produced in HEK293 cells with a purity exceeding 95%, as confirmed by silver staining and Western blot analysis, and was certified by the manufacturer to contain less than 0.1 EU/μg endotoxin. The lyophilized NS1 protein was reconstituted in sterile PBS to a stock concentration of 500 μg/mL and stored at −80°C until use. PBMCs were seeded in 24-well plates at a density of 5×10^5 cells per well in 500 μL of complete RPMI-1640 medium. For the JC-1 assay, round glass coverslips pre-coated with poly-L-lysine were placed at the bottom of each well to enhance cell adherence, and cells were allowed to adhere overnight at 37°C in a humidified atmosphere containing 5% CO_2. Following the initial culture period, cells from each donor were allocated to the experimental conditions. For the JC-1 assay, PBMCs from each donor were exposed to three conditions: untreated control (0 μg/mL NS1), recombinant NS1 at 1 μg/mL, and recombinant NS1 at 5 μg/mL. Each condition was assessed using three technical measurements within each donor. These technical measurements represented repeated measurements of the same biological sample and were not considered independent biological replicates. For Caspase-3 mRNA expression analysis, PBMCs from the same three donors were exposed to untreated control or recombinant NS1 at 1 μg/mL, with three technical PCR replicates performed for each donor and experimental condition. The exposure duration was 6 hours at 37°C in a humidified atmosphere containing 5% CO_2. Following exposure, cells were processed for JC-1 assessment or RNA isolation.

JC-1 staining was used to assess mitochondrial membrane potential-related cellular responses following NS1 exposure. JC-1 is a lipophilic cationic dye that accumulates in mitochondria in a membrane-potential-dependent manner. At relatively higher mitochondrial membrane potential, JC-1 forms red-fluorescent aggregates, whereas at relatively lower membrane potential it remains predominantly as green-fluorescent monomers. Following NS1 exposure, cells were washed twice with PBS and incubated with 2 μM JC-1 staining solution in complete RPMI-1640 medium for 15 minutes at 37°C in the dark. After incubation, the staining solution was removed and the cells were washed twice with PBS. Fluorescence was visualized using a Zoe Fluorescent Cell Imager (Bio-Rad, USA). Five predetermined standardized locations were evaluated for each well (center, 3 o'clock, 6 o'clock, 9 o'clock, and 12 o'clock positions) using brightfield and red fluorescence imaging (excitation/emission: 488/590 nm). For each field, the proportion of JC-1-positive cells among morphologically identified monocytes was calculated. The five field-level measurements were averaged to obtain a single donor-level value for each experimental condition. Monocytes were identified based on their characteristic morphological features observed in brightfield images. The resulting measurement represented the normalized proportion of JC-1-positive cells among morphologically identified monocytes and was not interpreted as a conventional red-to-green JC-1 fluorescence ratio.

Total RNA was extracted from PBMCs using the RNeasy Plus Mini Kit (Qiagen, Germany; Catalog No. 74134) according to the manufacturer's protocol. Following NS1 exposure and removal of the culture medium, cells were lysed directly in the 24-well plates by adding 350 μL of Buffer RLT Plus supplemented with β-mercaptoethanol (β-ME). Cell lysates were collected using a cell scraper, homogenized, and transferred to gDNA Eliminator spin columns to remove genomic DNA contamination. RNA was then purified using RNeasy spin columns following sequential washes with Buffer RW1 and Buffer RPE. Total RNA was eluted with 40 μL of RNase-free water and stored at −80°C until further analysis. RNA concentration and purity were determined using a NABI UV-Vis spectrophotometer by measuring absorbance at 260 and 280 nm. All RNA samples had A_{260}/A_{280} ratios between 1.8 and 2.1.

Gene expression analysis was performed using one-step quantitative real-time PCR (RT-qPCR) with the KAPA SYBR® FAST One-Step qRT-PCR Master Mix (Sigma-Aldrich, USA) on the AriaMx Real-Time PCR System (Agilent Technologies, USA). Each 20 μL reaction contained 10 μL of 2× KAPA SYBR Green Master Mix, 0.4 μL of KAPA RT Mix (50×), 0.4 μL each of forward and reverse primers (10 μM), 2 μL of template RNA (1 ng), and RNase-free water to adjust the final volume. The primer sequences used were as follows: GAPDH forward 5′-ACAACTTTGGTATCGTGGAAGG-3′ and reverse 5′-GCCATCACGCCACAGTTTC-3′; and Caspase-3 forward 5′-CTCCTAGCGGATGGGTGCTATTG-3′ and reverse 5′-TTATTAACGAAAACCAGAGCGCCG-3′. The thermal cycling protocol consisted of cDNA synthesis at 45°C for 15 minutes, enzyme activation at 95°C for 3 minutes, followed by 40 cycles of

denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 20 seconds. Melt curve analysis was performed to confirm primer specificity. Each donor-condition sample was analyzed in three technical PCR replicates. The three technical replicate Ct values were averaged to obtain a single Ct value for each donor and experimental condition before subsequent statistical analysis. Non-template controls and no-reverse-transcriptase controls were included in each run to verify the absence of contamination and genomic DNA amplification.

RT-qPCR data were analyzed with reference to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Ct values were determined using the AriaMx software with automatic baseline and threshold settings. For each donor and experimental condition, the mean Ct value obtained from the three technical PCR replicates was used for subsequent analysis. ΔCt values were calculated as Ct(target) − Ct(GAPDH). Relative Caspase-3 mRNA expression was initially explored using the comparative $2^{-}\Delta\Delta Ct$ method with GAPDH as the reference gene, with the corresponding untreated control condition used as the calibrator. However, substantial variation in GAPDH Ct values was observed between control and NS1-treated samples. Therefore, the resulting fold-change estimates were considered exploratory and were not interpreted as definitive normalized expression values. Statistical comparisons were performed using donor-level ΔCt values rather than technical replicate measurements. Primer amplification efficiency was assessed using a five-point standard curve for each primer pair, yielding efficiencies of 96.2% for GAPDH ($R^2 = 0.998$) and 95.5% for Caspase-3 ($R^2 = 0.996$), which were within the generally accepted range of 90–110%. Because the stability of GAPDH under NS1 exposure could not be adequately established, additional validation using multiple candidate reference genes, such as β-actin and 18S rRNA, is required in future studies.

Statistical analyses were performed using SPSS version 25.0 for Windows (SPSS Inc., Chicago, IL, USA). The three independent donors were considered biological replicates and constituted the experimental units (n = 3). Technical replicates were used to assess measurement variability and were not treated as independent observations. For the JC-1 assay, the five field-level measurements obtained from each donor were averaged to generate a single donor-level mean for each experimental condition. Thus, statistical analyses were performed using three donor-level observations per condition rather than individual fields or technical measurements. For RT-qPCR, the mean Ct values from the three technical PCR replicates were used to calculate donor-level ΔCt values. Because the control and NS1-treated samples were derived from the same donors, paired-samples *t*-tests were used for pairwise comparisons of donor-level data. A p-value < 0.05 was considered statistically significant. Given the small number of biological replicates (n = 3), all statistical findings were considered exploratory and should be confirmed in studies involving a larger number of independent donors.

RESULTS

JC-1 Staining

Monocyte cell counts were assessed by fluorescence microscopy using five standardized fields of view from each culture well. Measurements from the five fields were averaged to obtain a representative value for each donor and treatment condition. Data from three independent donors were analyzed as biological replicates (n = 3). The mean monocyte counts were 654.67 ± 154.53 cells/well in the control group, 636.33 ± 98.02 cells/well following exposure to NS1 at 1 µg/mL, and 754.33 ± 134.35 cells/well following exposure to NS1 at 5 µg/mL (Figure 1). These findings indicate that NS1 exposure did not substantially alter the number of monocytes under the experimental conditions.

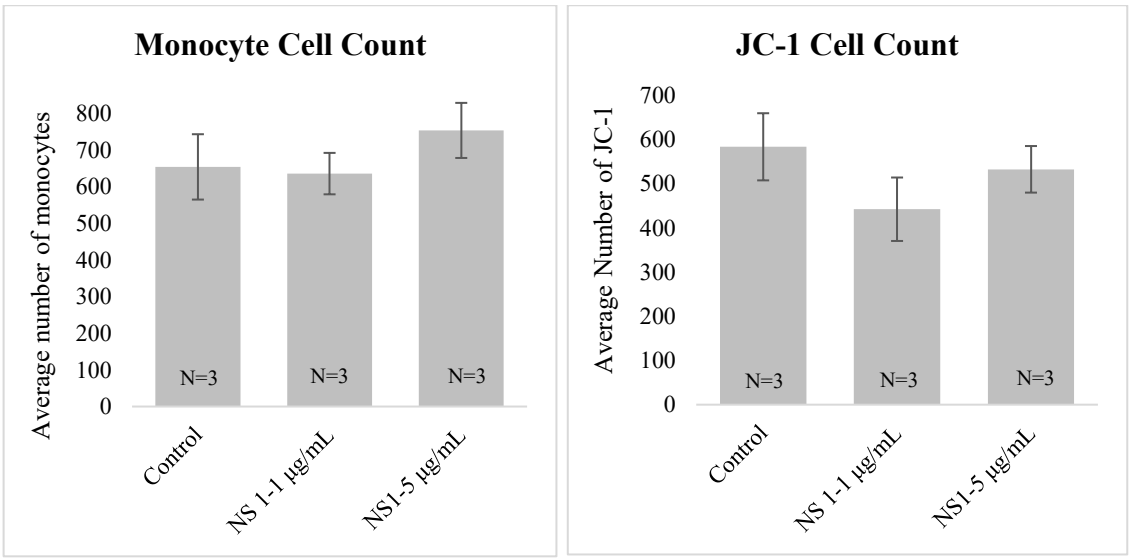


Figure 1. Number of Monocyte Cells and Cells with JC-1 Staining

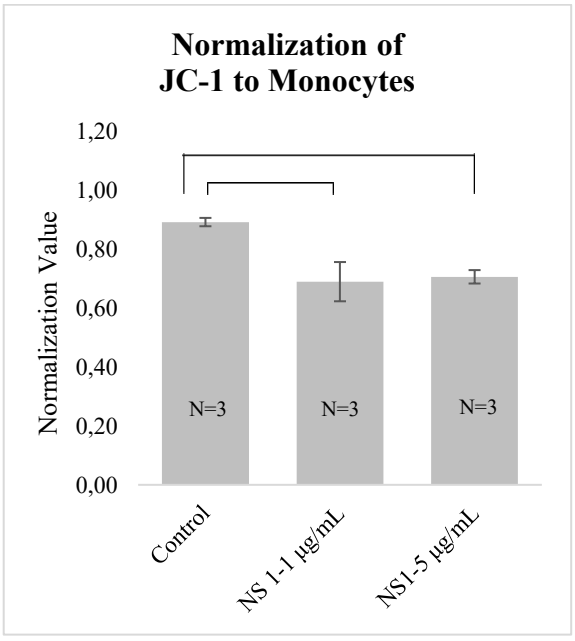


Figure 2. Proportion of JC-1-positive cells normalized to Monocyte Count

The number of JC-1-positive cells was subsequently assessed as an indicator of a mitochondrial membrane potential-related cellular response. The mean numbers of JC-1-positive cells were 583.67 ± 131.58 cells/well in the control group, 442.33 ± 124.43 cells/well in the NS1 1 µg/mL group, and 532.67 ± 91.31 cells/well in the NS1 5 µg/mL group (Figure 1). Although the NS1 1 µg/mL group showed a lower mean number of JC-1-positive cells than the control group, this observation should be interpreted descriptively given the small number of biological replicates.

To account for differences in monocyte numbers between culture wells, the number of JC-1-positive cells was normalized to the total number of morphologically identified monocytes. The resulting proportion of JC-1-positive cells was 0.892 ± 0.024 in the control group, 0.686 ± 0.104 in the NS1 1 µg/mL group, and 0.695 ± 0.031 in the NS1 5 µg/mL group (Figure 2). These values represent the proportion of JC-1-positive cells

among morphologically identified monocytes and were used as a normalized indicator of the mitochondrial membrane potential-related cellular response.

Because the three treatment conditions were obtained from the same independent donors, the donor was considered the experimental unit. Although the normalized proportion of JC-1-positive cells was lower in the NS1-treated groups, the overall Friedman test did not demonstrate a statistically significant difference among the three conditions ($p = 0.097$). Pairwise comparisons were considered exploratory because of the small number of biological replicates. No significant difference was observed between the NS1 1 µg/mL and 5 µg/mL groups ($t(2) = -0.220$, $p = 0.846$).

Comparison	t	df	p-value
Control vs NS1 1 ug/mL	3.130	2	0.089
Control vs NS1 5 ug/mL	8.062	2	0.015
NS1 1 µg/mL vs NS1 5 µg/mL	-0.220	2	0.846

Table 1. Pairwise comparasion of the normalize of JC-1 Positive

Given the small number of biological replicates (n = 3), these findings should be interpreted as preliminary evidence of an NS1-associated reduction in the proportion of JC-1-positive cells rather than definitive evidence of impaired mitochondrial membrane potential. The comparable values observed between the 1 µg/mL and 5 µg/mL NS1 groups suggest that increasing the NS1 concentration from 1 to 5 µg/mL did not result in an additional reduction in the normalized JC-1-positive cell proportion. Therefore, the 1 µg/mL concentration was selected for subsequent RT-qPCR analysis of Caspase-3 mRNA expression.

RT-qPCR

Total RNA concentration was assessed for the control and NS1 1 µg/mL groups. The mean RNA concentrations were 3.13 ± 0.15 and 3.73 ± 0.12, respectively (Figure 3). Because samples from the same three donors were compared between treatment conditions, the donor was considered the experimental unit. The difference in RNA concentration was evaluated at the donor level. Given the small number of biological replicates (n = 3), these findings were interpreted descriptively and primarily used to characterize the RNA samples for subsequent RT-qPCR analysis (Figure 3).

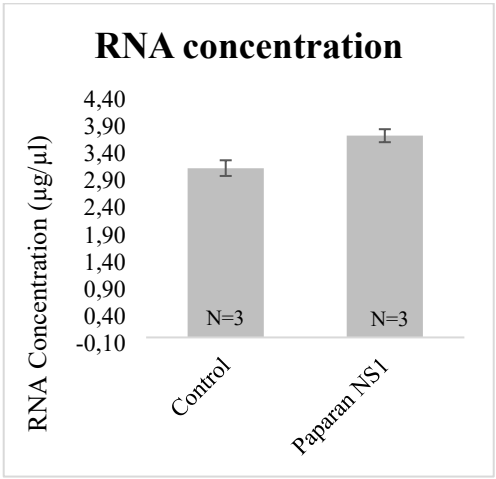


Figure 3. Total RNA Control vs Treatment

Analysis of GAPDH GAPDH Ct values were 28.17 ± 0.19 in the control group and 25.13 ± 0.30 in the NS1 1 µg/mL group (Figure 4). The lower Ct values observed following NS1 exposure indicated increased

GAPDH transcript abundance under the treatment condition. Because the GAPDH Ct values differed substantially between control and NS1-treated samples, GAPDH was considered potentially unstable as a reference gene under the experimental conditions. Therefore, these data raised concerns regarding the validity of GAPDH-based normalization of Caspase-3 mRNA expression. Caspase-3 Ct values were 31.37 ± 0.24 in the control group and 24.58 ± 0.70 in the NS1 1 µg/mL group (Figure 4). Because Ct values alone do not account for differences in reference gene abundance, Caspase-3 mRNA expression was evaluated using donor-level ΔCt values. The paired-samples t-test showed a significant difference in ΔCt between the control and NS1-treated groups ($t(2) = 11.85$, $p = 0.007$). (Figure 4). However, because GAPDH showed substantial variation between treatment conditions, the observed ΔCt difference should be interpreted cautiously. These findings suggest a difference in Caspase-3 transcript abundance between control and NS1-treated PBMCs; however, they do not provide definitive evidence of increased Caspase-3 mRNA expression without validation using stable reference genes. Confirmation using a validated normalization strategy, together with protein-level or functional apoptosis assays, is required.

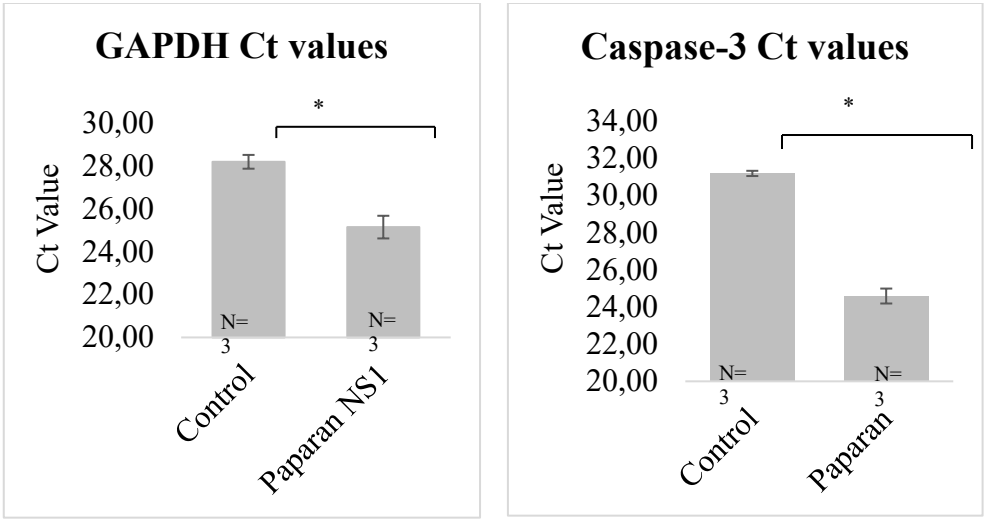


Figure 4. Ct values of GAPDH and Caspase-3 in control and NS1-treated PBMCs

The RT-qPCR data were analyzed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The cycle threshold (Ct) values were determined using the AriaMx software (Agilent Technologies, USA) with automatic baseline and threshold settings. For each sample, the average Ct value was calculated from three technical replicates. The relative expression analysis was initially explored using the $2^{-}\Delta\Delta Ct$ method with GAPDH as the reference gene. However, substantial differences in GAPDH Ct values between control and NS1-treated samples indicated that GAPDH may not be stable under the experimental conditions. Therefore, the GAPDH-normalized fold-change calculation was considered exploratory rather than definitive. Donor-level ΔCt values were used for statistical comparison, while definitive relative expression analysis requires validation using one or more stable reference genes.

RT-qPCR analysis showed a difference in Caspase-3 transcript abundance between control and NS1-treated PBMCs based on donor-level ΔCt analysis ($t(2) = 11.85$, $p = 0.007$). An exploratory GAPDH-normalized analysis suggested an apparent increase in Caspase-3 mRNA mRNA expression; however, this estimate should be interpreted cautiously because GAPDH stability under NS1 exposure was not established. However, because GAPDH Ct values differed substantially between control and NS1-treated samples, the stability of GAPDH as a reference gene could not be established. Consequently, the calculated fold change should be considered exploratory and should not be interpreted as definitive evidence of Caspase-3 transcriptional upregulation. Further validation using stable reference genes and protein-level or functional apoptosis assays is required.

Primer amplification efficiency was assessed using a five-point standard curve generated from 10-fold serial dilutions of pooled RNA. The amplification efficiencies were 96.2% for GAPDH ($R^2 = 0.998$) and

95.5% for Caspase-3 ($R^2 = 0.996$), both within the generally accepted range of 90–110%. However, GAPDH Ct values differed substantially between control and NS1-treated samples, limiting the reliability of GAPDH-based normalization. Validation using additional reference genes, such as β-actin and 18S rRNA, is required before definitive relative expression analysis can be performed.

DISCUSSION

The present study investigated the effects of recombinant DENV-2 NS1 protein on mitochondrial membrane potential-related cellular responses and Caspase-3 mRNA expression in human PBMCs. NS1 exposure did not significantly alter monocyte cell numbers, but the proportion of JC-1-positive cells was lower in NS1-treated groups than in the control group. These findings suggest that NS1 exposure may be associated with alterations in mitochondrial membrane potential-related cellular responses. However, because JC-1 staining in this study was assessed as the proportion of JC-1-positive cells rather than the conventional red-to-green fluorescence ratio, the findings should not be interpreted as a direct quantitative measurement of mitochondrial membrane potential.

RT-qPCR analysis showed a significant difference in donor-level ΔCt values for Caspase-3 between control and NS1-treated PBMCs. An exploratory GAPDH-normalized analysis suggested an apparent increase in Caspase-3 mRNA expression; however, this estimate should be interpreted cautiously because GAPDH stability under NS1 exposure was not established. Therefore, the calculated fold change should be considered exploratory and cannot provide definitive evidence of Caspase-3 mRNA upregulation without validation using stable reference genes.

The present findings also do not establish Caspase-3 activation or apoptosis, because only Caspase-3 mRNA expression and JC-1-associated cellular responses were assessed. Direct confirmation using cleaved Caspase-3 protein, caspase activity, Annexin V/PI, TUNEL, or other functional apoptosis assays is required. Furthermore, the small number of independent donors (n = 3), use of healthy-donor PBMCs, short exposure period, and morphological identification of monocytes limit the generalizability and interpretation of the findings.

Overall, these results provide preliminary evidence that DENV-2 NS1 exposure is associated with changes in mitochondrial membrane potential-related cellular responses and apoptosis-related gene expression in human PBMCs. Further studies using larger numbers of biological replicates, validated reference genes, direct mitochondrial membrane potential measurements, and complementary apoptosis assays are needed to determine whether these changes represent a direct apoptotic effect of NS1.

CONCLUSION

Recombinant DENV-2 NS1 exposure was associated with a reduction in the proportion of JC-1-positive cells and differences in Caspase-3 transcript abundance in human PBMCs. These findings provide preliminary evidence of alterations in mitochondrial membrane potential-related cellular responses and apoptosis-associated gene expression following NS1 exposure. However, the substantial variation in GAPDH expression limits the interpretation of GAPDH-normalized Caspase-3 mRNA expression, and the present findings do not establish Caspase-3 activation or apoptosis directly. Further studies using validated reference genes, direct assessment of mitochondrial membrane potential, and complementary protein-level and functional apoptosis assays are required to confirm these findings and clarify the role of DENV-2 NS1 in PBMC responses.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest related to this manuscript. The authors have no financial, commercial, or personal relationships with any company whose products are relevant to this study or with companies producing competing products. No financial arrangements or other potential conflicts of interest exist that could have influenced the design, conduct, analysis, or reporting of this study.

ACKNOWLEDGMENTS

The authors would like to express their sincere appreciation and gratitude to Mr. Zukhrofi Muzar, Mrs. Nurfadly, Mrs. Siti Masliana Siregar, and Mr. Shahrul Rahman for their contributions and support during the conduct of this study. The authors also wish to thank the Integrated Laboratory of the Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Sumatera Utara (FKIK UMSU) for providing technical support and facilities. This research was funded by FKIK UMSU.

DATA AVAILABILITY

The data supporting the findings of this study are not publicly available due to privacy and ethical considerations related to human biological samples. However, the data generated and analyzed during this study are available from the corresponding author upon reasonable request and subject to appropriate ethical and confidentiality requirements.

AUTHOR CONTRIBUTIONS

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Riyanda Indrawan Sani	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓	✓
Nurfadly		✓		✓	✓		✓			✓		✓		
Zukhrofi Muzar	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
Shahrul Rahman				✓	✓					✓		✓		
Siti Masliana Siregar		✓		✓	✓					✓		✓		✓

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

LIST OF ABBREVIATIONS

β-ME: Beta-Mercaptoethanol; DENV: Dengue Virus; DNA: Deoxyribonucleic Acid; FBS: Fetal Bovine Serum; GBS: Guillain-Barré Syndrome; HEK293: Human Embryonic Kidney 293 Cells; JC-1: 5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethylbenzimidazolylcarbocyanine Iodide; NS1: Non-Structural Protein 1; PBS: Phosphate-Buffered Saline; RLT: RNA Lysis Tissue Buffer; RNA: Ribonucleic Acid; RPE: RNA Protecting Elution Buffer; RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction; RW1: RNA Wash Buffer 1.

ETHICAL APPROVAL

This study involving human participants was conducted in accordance with all relevant national regulations and institutional policies and followed the principles of the Declaration of Helsinki and its subsequent amendments. The study protocol was reviewed and approved by the Health Research Ethics Committee of Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia (Ethical Approval No. 1359/KEPK/FKUMSU/2024). Written informed consent was obtained from all participants prior to blood sample collection and participation in the study.

REFERENCES (10 PT)

1. Du M, Jing W, Liu M, Liu J. The Global Trends and Regional Differences in Incidence of Dengue Infection from 1990 to 2019: An Analysis from the Global Burden of Disease Study 2019. Infect Dis Ther 2021;10(3):1625–1643.
2. Bhatt S, Gething PW, Brady OJ, et al. The global distribution and burden of dengue. Nature 2013;496(7446):504–7.
3. Sudaryanto A, Liao W-C, Lin Y-P, Ho W-C. Two Decades of Dengue in Indonesia: Long-Term Trends in Incidence, Mortality, and Disability-Adjusted Life Years, 2005-2024. Pathogens 2026;15(4).

4. Prayitno A, Taurel A-F, Nealon J, et al. Dengue seroprevalence and force of primary infection in a representative population of urban dwelling Indonesian children. PLoS Negl Trop Dis 2017;11(6):e0005621.
5. Rodrigo C, Sigera C, Fernando D, Rajapakse S. Plasma leakage in dengue: a systematic review of prospective observational studies. BMC Infect Dis 2021;21(1):1082.
6. Li G-H, Ning Z-J, Liu Y-M, Li X-H. Neurological Manifestations of Dengue Infection. Front Cell Infect Microbiol 2017;7:449.
7. Glasner DR, Puerta-Guardo H, Beatty PR, Harris E. The Good, the Bad, and the Shocking: The Multiple Roles of Dengue Virus Nonstructural Protein 1 in Protection and Pathogenesis. Annu Rev Virol 2018;5(1):227–253.
8. Wanigarathna US, Rajapakse S, Pathirana SL, Handunnetti SM, Nitsche A, Fernando N. Dengue NS1 as a Driver of Immune-Mediated Pathogenesis. Trop Med Infect Dis 2026;11(5).
9. Puerta-Guardo H, Glasner DR, Harris E. Dengue Virus NS1 Disrupts the Endothelial Glycocalyx, Leading to Hyperpermeability. PLoS Pathog 2016;12(7):e1005738.
10. Trivedi S, Chakravarty A. Neurological Complications of Dengue Fever. Curr Neurol Neurosci Rep 2022;22(8):515–529.
11. Calderón-Peláez M-A, Velandia-Romero ML, Bastidas-Legarda LY, Beltrán EO, Camacho-Ortega SJ, Castellanos JE. Dengue Virus Infection of Blood–Brain Barrier Cells: Consequences of Severe Disease. Front Microbiol 2019;10.
12. Kulkarni R, Pujari S, Gupta D. Neurological Manifestations of Dengue Fever. Ann Indian Acad Neurol 2021;24(5):693–702.
13. Saisingha K, Jearanaiwitayakul T, Watterson D, Modhiran N, Ponpuak M, Ubol S. Transcriptome Analysis of Monocytes Treated With Dengue Virus Nonstructural Protein 1 Revealed a Shift in Transcripts Involved in Self-Propagated Proinflammation and Antiviral Responses. J Infect Dis 2025;231(6):e1170–e1182.
14. Dayarathna S, Senadheera B, Jeewandara C, et al. Dengue NS1 interaction with lipids alters its pathogenic effects on monocyte derived macrophages. J Biomed Sci 2024;31(1):86.
15. Wong KL, Chen W, Balakrishnan T, Toh YX, Fink K, Wong S-C. Susceptibility and response of human blood monocyte subsets to primary dengue virus infection. PLoS One 2012;7(5):e36435.
16. Srikiatkhachorn A, Wichit S, Gibbons R V, et al. Dengue viral RNA levels in peripheral blood mononuclear cells are associated with disease severity and preexisting dengue immune status. PLoS One 2012;7(12):e51335.
17. Torrentes-Carvalho A, Azeredo EL, Reis SRI, et al. Dengue-2 infection and the induction of apoptosis in human primary monocytes. Mem Inst Oswaldo Cruz 2009;104(8):1091–9.
18. Malavige GN, Huang L-C, Salimi M, Gomes L, Jayaratne SD, Ogg GS. Cellular and cytokine correlates of severe dengue infection. PLoS One 2012;7(11):e50387.
19. Qi Y, Li Y, Zhang Y, et al. IFI6 Inhibits Apoptosis via Mitochondrial-Dependent Pathway in Dengue Virus 2 Infected Vascular Endothelial Cells. PLoS One 2015;10(8):e0132743.
20. Chatel-Chaix L, Cortese M, Romero-Brey I, et al. Dengue Virus Perturbs Mitochondrial Morphodynamics to Dampen Innate Immune Responses. Cell Host Microbe 2016;20(3):342–356.
21. Ahmed S, Varga RD, Yang J. The Impacts of Dengue Virus Infection on Mitochondrial Functions and Dynamics. Int J Mol Sci 2025;26(18).
22. Muller DA, Young PR. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antiviral Res 2013;98(2):192–208.