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Research paper

Drug repurposing of N-acetyl cysteine as antiviral against dengue virus infection

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ABSTRACT

Liver injury is one of the hallmark features of severe dengue virus (DENV) infection since DENV can replicate in the liver and induce hepatocytes to undergo apoptosis. N-acetyl cysteine (NAC), which is a clinically-used drug for treating acetaminophen toxicity, was found to benefit patients with DENV-induced liver injury; however, its mechanism of action remains unclear. Accordingly, our aim was to repurpose NAC in the preclinical studies to investigate its mechanism of action. Time of addition experiments in HepG2 cells elucidated effectiveness of NAC to reduce infectious virion at pre-, during- and post infection. In DENV-infected mice, NAC improved DENV-associated clinical manifestations, including leucopenia and thrombocytopenia, and reduced liver injury and hepatocyte apoptosis. Interestingly, we discovered that NAC significantly reduced DENV production in HepG2 cells and in liver of DENV-infected mice by induction of antiviral responses via interferon signaling. NAC treatment in DENV-infected mice helped to maintain antioxidant enzymes and redox balance in the liver. Therefore, NAC reduces DENV production and oxidative damage to ameliorate DENV-induced liver injury. Taken together, these findings suggest the novel therapeutic potential of NAC in DENV-induced liver injury and recommend evaluating its efficacy and safety in humans with DENV-induced liver injury.

1. Introduction

Dengue virus (DENV) infection is one of several arboviral diseases that affect humans that are accelerating their spread into and across the tropical and subtropical regions of the world (Murray et al., 2013). Patients with DENV infection can develop any one of a range of disease severities, including self-controlling comparatively mild dengue fever (DF), dengue hemorrhagic fever (DHF), or the most severe dengue shock syndrome (DHS). Although all 4 of the DENV serotypes (serotypes 1–4) are-known to cause disease, the severity of disease was reported to differ among serotypes and strains (Fox et al., 2011; Vicente et al., 2016). The disease pathogenesis of DENV infection is complex with serotype specificity (Lin et al., 2011; Martina, 2014), and is still being investigated.

Liver injury was evident in DENV-infected patients (Samanta and Sharma, 2015), and was reported to be one of the major disease criteria for the severe forms of DENV infection by the World Health Organization (WHO) (Jayaratne et al., 2012). The clinical pathology of liver injury has been studied in DENV infection, and virus replication was found to be one of the factors that contributes most to liver impairment (Franca et al., 2010). In an immunocompetent mouse model of DENV infection exhibiting liver injury, inhibitors of mitogen-activated protein kinases (MAPKs) limited hepatic cell apoptosis and reduced liver injury in DENV-infected mice; however, these inhibitors were not able to restrict virus replication in the liver (Sreekanth et al., 2014, 2016, 2017, 2018).

Drug repurposing in the advancement of dengue antiviral development was recently reviewed (Low et al., 2018). In the present study, N-acetyl cysteine (NAC), a United States Food and Drug Administration (US FDA)-approved mucolytic drug and dietary supplement, is widely used as an antioxidant (Mokhtari et al., 2017), and to treat acetaminophen (also known as paracetamol) overdose (Yoon et al., 2016)

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was repurposed against DENV-induced liver injury. Two cohort studies reported complete recovery of fulminant liver failure and massive bleeding in severe DENV-infected patients after treatment with intravenous NAC infusion (Kumarasena et al., 2010; Manoj et al., 2014). However, the mechanism of action underlying this therapeutic effect was not addressed in either of those two studies. Recently, Dissanayake, et al. highlighted the need to identify the mechanism by which NAC reverses DENV-induced liver injury (Dissanayake and Seneviratne, 2018). We repurposed NAC in the preclinical studies to identify its mechanism of action in DENV-infected HepG2 cells, and in an immunocompetent mouse model of DENV-induced liver injury.

2. Materials and methods

2.1. Cell cultures, virus, and treatment

Human hepatocarcinoma cell lines (HepG2 cells; ATCC, Manassas, VS, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin-streptomycin at 37 °C in a humidified chamber with 5% CO_2 incubation. NAC was dissolved in 0.02% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation, St. Louis, MO, USA). HepG2 cells were seeded and infected with DENV serotype-2, strain-16681 at a MOI of 5, and then treated with vehicle or NAC at concentrations of 5 mM, 10 mM, and 20 mM.

2.2. Determination of cytotoxicity and IC_{50}

Cell viability assay was conducted using PrestoBlue® Cell Viability Reagent (Invitrogen, CA, USA). Experiments were conducted to determine the effect of NAC in the cell viability of both uninfected and DENV-infected HepG2 cells. Briefly, PrestoBlue reagent was added to both uninfected and DENV-infected HepG2 cells, those were treated with NAC at different concentrations including 5 mM, 10 mM and 20 mM in a 96-well assay plate. The plate was allowed to incubate for 30 min in the dark and the absorbance was measured at 570/595 nm. For the uninfected HepG2 cells, the percentage cell viability of NAC treatments was normalized to that of untreated HepG2 cells. For the DENV-infected HepG2 cells, the percentage cell viability of NAC treatment was normalized to that of mock-infected HepG2 cells.

NAC was serially diluted and treated with the DENV-infected HepG2 cells. An untreated DENV-infected HepG2 cells was maintained as the control group. The plates were maintained in a 37 °C CO_2 incubator for 48 h post-infection. The percentage intracellular DENV E antigen in the NAC treated groups and untreated control group was determined. The concentration that reduced the 50% intracellular DENV E antigen (referred as IC_{50} value) was calculated.

2.3. Effect of NAC during the steps of DENV life cycle

HepG2 cells were infected with DENV-2 at an MOI of 5 and withdrawn 2 h post-infection. The cells were then treated with vehicle or NAC at concentrations of 5 mM, 10 mM, and 20 mM. The cells were collected after 48 h post-infection and experiments were conducted to define the effect of NAC on the viral life cycle including viral RNA synthesis, protein synthesis, and virion production and exocytosis.

A real-time reverse transcription PCR (Real-time RT PCR) was conducted to determine the effect of NAC treatment in the viral RNA synthesis step of viral life cycle. The primer set used for DENV E is shown in the Table 1.

An enzyme-linked immunosorbent assay (ELISA) was used to determine the intracellular DENV E protein. The cells were stained with mouse monoclonal antibody to DENV E protein (4G2 clone) and horseradish (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody (Dako, CA, USA) at 48 h post-treatment. A substrate (3,3′,5,5′-tetramethylbenzidine; Invitrogen, CA, USA) was added and the absorbance was measured at 430/630 nm.

Western blot analysis was conducted to observe the effect of NAC in the expression of DENV E protein. Equal concentrations of proteins obtained from the cell lysates were loaded and separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin for 1 h, allowed to incubate with a mouse monoclonal primary antibody to DENV E protein (4G2 clone) for 1 h. The membrane was washed and incubated over night at 4 °C with horseradish (HRP)-conjugated rabbit anti-mouse immunoglobulin antibody. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to enhance the signal from immune complexes. GAPDH was used as the housekeeping gene control. Densitometry analysis was conducted using ImageJ software (United States National Institutes of Health, Bethesda, MD, USA).

Supernatants were collected from the cells and undergone standard FFU assay protocol (Jirakanjanakit et al., 1997). The supernatants were added to Vero cells and incubated for 2 h followed by overlaying with 1.5% carboxymethyl cellulose (CMC) in minimal essential medium (MEM) for 72 h. The cells were thoroughly washed with PBS, fixed with 3.7% formaldehyde, and permeabilized with 1% Triton X-100. Further, the cells were incubated with the primary antibody to DENV E (4G2 clone) for 1 h and with HRP-conjugated rabbit anti-mouse secondary antibody (Dako) for 30 min. The 3,3′-diaminobenzidine substrate (Sigma Aldrich) was used to observe the foci of infected cells. The foci were counted under a light microscope and the FFU was calculated as the number of foci.

2.4. Time of addition assay

In a 96-well plate, HepG2 cells were infected with DENV-2 at an MOI of 5 and were treated with 5 mM, 10 mM and 20 mM concentrations of NAC at different points of time including pre-, during- and post-infection. For the pre-treatment experiments (−2 h), HepG2 cells were incubated with NAC for 2 h, and washed out, and DENV-2 infection was quickly initiated. For the during experiments (0 h), NAC and DENV-2 were added together to HepG2 cells for 2 h and the media was removed, cells were washed and further incubated. For the post-treatment experiments, HepG2 cells were infected with DENV-2 for 2 h and treated with NAC at 2, 6, 12 and 24 h to post-infection in separate wells of the plate. DENV-2 infected HepG2 cells those were untreated was also maintained as the control group. The plate was maintained in a 37 °C CO_2 incubator for 48 h to post-infection and FFU assay was conducted with the obtained supernatants, using the protocol mentioned in 2.3.

2.5. Effect of NAC in HepG2 cells infected with different MOI of DENV-2

HepG2 cells were infected with varying MOI of DENV-2 including MOI-1 and MOI-10. Treatment with NAC at concentrations of 5 mM, 10 mM, and 20 mM were given 2 h post-infection. The cells were kept in a 37 °C CO_2 incubator for 48 h post-infection and FFU assay was conducted using the protocol mentioned in 2.3, to determine the effect of NAC in the different MOI of DENV-2.

2.6. Effect of NAC in the HepG2 cells infected with different serotypes of DENV

HepG2 cells were infected with serotypes of DENV including DENV-1 (strain Hawaii), DENV-3 (strain H87) and DENV-4 (strain H241) at an MOI of 5, and then treated with vehicle or NAC at concentrations of 5 mM, 10 mM, and 20 mM for 2 h post-infection. The cells were kept in a 37 °C CO_2 incubator for 48 h post-infection and FFU assay was conducted using the protocol mentioned in 2.3, to determine the effect of NAC with different serotypes of DENV.

Table 1
Primer design used for DENV-E and DENV-NS1.

No	Gene	Forward primer	Reverse primer
1	*DENV-E*	ATCCAGATGTCATCAGGAAAC	CCGGCTCTACTCCTATGATG
2	*DENV-NS1*	CCGGCCAGATCTGGAGACATCAAAGGAATC	GCCATCAATGAGAAAGGTCTGG

2.7. Murine DENV infection and treatments

Eight-week-old male BALB/c mice were purchased from the National Laboratory Animal Centre (NLAC), Mahidol University, Thailand, and the mice were maintained under pathogen-free conditions at the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Mice were intravenously infected with 4×10^5 FFU of DENV-2 (strain-16681) via the lateral tail vein. Treatments were introduced via the same route at three time points; 1 h before, and 1 h and 24 h after DENV infection. NAC (Sigma-Aldrich) at a dose of 150 mg/kg and the JNK inhibitor SP600125 (Abcam, Cambridge, United Kingdom) at a dose of 20 mg/kg prepared in 2% DMSO (v/v) were used for administration. Control groups of mice were maintained, including DENV-infected mice treated with 2% DMSO, and uninfected mice treated with 2% DMSO. Mice were euthanized with an intraperitoneal injection of sodium pentobarbital. Blood sample was collected on day 3 and day 7 post-infection and the liver tissue samples were harvested on day 7 just before the sacrifice. The total body weight and the weight of the liver were measured using a calibrated balance scale.

The experimental protocol was approved by the Siriraj Animal Care and Use Committee (SI-ACUP 025/2557) in accordance with the ethical principles and guidelines of National Research Council (NRC) of Thailand, and the Siriraj Biosafety Risk Management Taskforce, Faculty of Medicine Siriraj Hospital, Mahidol University (SI-2015-001).

2.8. Hematology, liver transaminases, and histopathology analysis

Murine blood samples were collected in vacutainer tubes containing EDTA for the hematology analysis, and rapidly analyzed with a CELL-DYNTM 3700 hematological auto analyzer (Abbott, IL, USA). For the serum, the blood samples were allowed to clot and then centrifuged. For the histopathology analysis, the liver tissues were fixed in 10% formalin, paraffin embedded, sectioned, and mounted for hematoxylin and eosin (H&E) staining.

2.9. DENV-NS1 viral RNA quantification and FFU assay

Total RNA was extracted from the liver tissues and serum samples with the Invitrap Spin Universal RNA Mini Kit (Stratec Molecular). DENV-NS1 viral RNA was quantified by qRT-PCR in a Roche Light Cycler 480, using a previously reported protocol (Sreekanth et al., 2014). Briefly, an *in vitro* transcription derived DENV-NS1 RNA with known copy number was serially diluted and allowed to qRT-PCR in the presence of SYBR Green I reaction mix (Roche) and NS1 specific primers (the primer design is shown in the Table .1). The obtained Ct values of the standard were used to plot a standard curve. The Ct values of the viral standards were compared with the samples to determine the DENV-NS-1 viral copies. FFU assay was performed using filtered and sterilized supernatants obtained from liver tissue homogenates using the protocol mentioned in 2.3.

2.10. SOD and catalase activity assays

Superoxide dismutase (SOD) activity assay (catalog no. ab65354) and catalase (CAT) activity assay (catalog no. ab83464) were both obtained from Abcam. Liver tissues were homogenized and centrifuged for 5 min at 4 °C at 14,000 g. The obtained supernatants were collected in fresh tubes, and the experiments were conducted according to the manufacturer's instructions. Using a Synergy microplate reader, the absorbance of the SOD and CAT reaction mixtures was read at 450 nm and 570 nm, respectively.

2.11. Antiviral gene expression analysis

Total RNA was extracted from the cell lysate (High Pure RNA Isolation Kit, Roche, Mannheim, Germany) or liver tissues (Invitrap Spin Universal RNA Mini Kit, Stratec Molecular) and converted to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA, USA).

For the real-time RT-PCR array, cDNA was mixed with SYBR Green RT^2 qPCR Mastermix (QIAGEN, Hilden, Germany), and then aliquoted into an RT^2 Profiler™ PCR Array (Qiagen) containing 84 selected antiviral genes. PCR amplification was performed in a Roche LightCycler 480 Instrument (Roche Applied Science, Penzberg, Germany), and Ct values were analyzed using the 'Qiagen Data Analysis Centre' web platform ($2^{-\Delta\Delta Ct}$ analysis).

For the real-time RT-PCR analysis, the cDNA was allowed to mix with LightCycler® 480 SYBR Green Mastermix (Invitrogen), and primer set for each individual gene of interest. The primer design for the human (Table 2) and mouse (Table 3) is separately shown. Reactions were performed in a Roche LightCycler 480 Instrument, and Ct values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the house-keeping gene control. The results were further analyzed by $2^{-\Delta\Delta Ct}$ method, and the expressions are reported as fold increase or decrease.

2.12. Western blot analysis

Total proteins were extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membrane, and blocked to prevent non-specific binding. Membranes were incubated overnight with rabbit anti-total JNK1/2 or

Table 2
Primer design used for antiviral gene expression analysis (human).

No.	Gene	Forward primer	Reverse primer
1	*RIG-1*	AGTGAGCATGCACGAATGAA	GGGATCCCTGGAAACACTTT
2	*MDA-5*	CTCAGGCCTTACCAAATGGA	TCCAGGCTCAGATGCTTTTT
3	*IFN-α*	TTTCTCCTGCCTGAAGGACAG	GCTCATGATTTCTGCTCTGAC
4	*IFNAR1*	AAAATGGCAATGATAGG	CAGGCTGAGCAGAAGG
5	*IFN-β*	GACGCCGCATTGACCATCTA	TTGGCCTTCAGGTAATGCAGAA
6	*OAS1*	CTCAAGAGCCTCATCCG	GCAGAGTTGCTGGTAGTTTA
7	*OAS2*	GCTTTGATGTGCTTCCTGCCTT	ACCCCTTTGGCTTCAGTTTCCTT
8	*GAPDH*	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATCGCAACTG

Table 3
Primer design used for the gene expression analysis (mouse).

No.	Gene	Forward primer	Reverse primer
1	*RIG-1*	AAGGAAAACTGGCCAAAGGT	TGGTTTCAATGGGCTGTGTA
2	*MDA-5*	GCTGCTAAAGACGGAAATCG	TCTTGTCGCTGTCATTGAGG
3	*IFNA2*	TCTGTGCTTTCCTCGTGATG	TTGAGCCTTCTGGATCTGCT
4	*IFNAR1*	GGTGGTTCTGTCTCGGTGTT	GCCAGCTCCTCCAGTTAGTG
5	*IFNB1*	CCCTATGGAGATGACGGAGA	CTGTCTGCTGGTGGAGTTCA
6	*IRF-7*	GAAGACCCTGATCCTGGTGA	CCAGGTCCATGAGGAAGTGT
7	*MAVS*	AGAGCAACTCCTCCAGACCA	AACGGTTGGAGACACAGGTC
8	*OAS-2*	AGCCTTGGAAAGTGCCAGTA	CTGCCAGAGAGGACTGAACC
9	*TLR-3*	ATATGCGCTTCAATCCGTTC	CAGGAGCATACTGGTGCTGA
10	*GAPDH*	TGAATACGGCTACAGCAACA	AGGCCCCTCCTGTTATTATG

mouse anti-phosphorylated JNK1/2 or rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, USA). The membrane was further incubated in the dark at room temperature with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Dako Denmark A/S, Glostrup, Denmark). Immune complexes were detected by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Waltham, MA, USA). GAPDH was used as the housekeeping gene control. Densitometry analysis was conducted using ImageJ software (United States National Institutes of Health, Bethesda, MD, USA).

2.13. Statistical analysis

Study data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. N-acetyl cysteine (NAC) interferes the life cycle of DENV

Preliminarily, experiments were conducted to examine the cytotoxicity of NAC in HepG2 cell line without DENV infection. We did not observe any significant change in the cell viability, when NAC at 5 mM, 10 mM and 20 mM were treated with HepG2 cells (Fig. 1A). However, NAC reduced DENV infection. IC_{50} value of NAC in the DENV-infected HepG2 cells was determined. The concentration that reduced 50% of the intracellular DENV E antigen was found to be 3.667 mM (Fig. 1B).

The effect of NAC treatment during different stages of the DENV life cycle was investigated. During the viral RNA synthesis step, we found that 20 mM of NAC treatment impeded the intracellular viral RNA (Fig. 2A). A cell-based ELISA was performed to analyze the expression of DENV E antigen. NAC treatment at different concentrations exhibited significant reduction in DENV E protein expression in a dose-dependent manner (Fig. 2B). Western blot analysis revealed similar results when DENV-infected HepG2 cells were treated with NAC (Fig. 2C and D). A FFU assay was conducted to investigate the effect of NAC in the exocytosis step of virus replication. Our results suggest that NAC reduces the number of infectious virions in DENV-infected HepG2 cells in a dose-dependent manner (Fig. 2E). Besides, the effect of NAC in the cell viability of DENV-infected HepG2 cells was determined. HepG2 cells infected with DENV-2 showed a reduction in cell viability and NAC treatment in DENV-infected HepG2 cells dose dependently restored this reduction of cell viability (Fig. 2F).

Time-of addition assay was conducted to identify the effective antiviral activity of NAC on different time points that characterizing the stages of DENV life cycle. The graphical representation of the experimental design is shown in Fig. 3A. Our finding suggests NAC reduced DENV-2 infectivity in HepG2 cells at pre-, during- and post infection with concentrations of 5 mM (Fig. 3B), 10 mM (Fig. 3C) and 20 mM (Fig. 3D). The DENV-2 infectivity was reduced when NAC treatment was given simultaneously with DENV-infection (during-) for 2 h; suggesting NAC interferes the DENV entry. Similar results were seen at 2 and 6 h post-infection suggesting the efficacy of NAC in the viral translation step. The maximum reduction in the DENV-2 infectivity was observed with NAC at 20 mM concentration at 6 h post infection suggesting NAC is highly effective at the RNA synthesis step of DENV life cycle (Fig. 3D). We also observed reduction in DENV-2 infectivity at 12 h post infection suggesting NAC reduced the production of virus progeny and the exocytosis stage of DENV life cycle.

3.2. NAC reduced DENV-2 infectivity with different multiplicity of infection (MOI)

Experiments were conducted to identify the efficacy of NAC (5 mM, 10 mM, and 20 mM) in HepG2 cells infected with varying MOI of DENV-2 including MOI-1 and MOI-10. We observed significant reduction in the virus titer at MOI-1 (Fig. 4A) and MOI-10 (Fig. 4B) with the different dosages of NAC suggesting its potency of NAC to moderate DENV burden at different MOI.

3.3. NAC reduced infectivity in HepG2 cells infected with the different serotype of DENV

Experiments were conducted to identify the efficacy of NAC (5 mM, 10 mM, and 20 mM) in HepG2 cells infected with different serotypes of DENV (including DENV-1, DENV-3 and DENV-4) at MOI-5. We observed significant reduction in the virus titer with the dosages of NAC used in HepG2 cells infected with DENV-1 (Fig. 5A), DENV-3 (Fig. 5B) and DENV-4 (Fig. 5C). Our result suggests the competency of NAC to reduce DENV infection in different serotypes.

3.4. NAC exerted antiviral responses to suppress DENV replication

To explain the antiviral effects of NAC in DENV-infected HepG2 cells, real-time polymerase chain reaction (RT-PCR) analysis was performed for the major antiviral genes. NAC treatment in DENV-infected

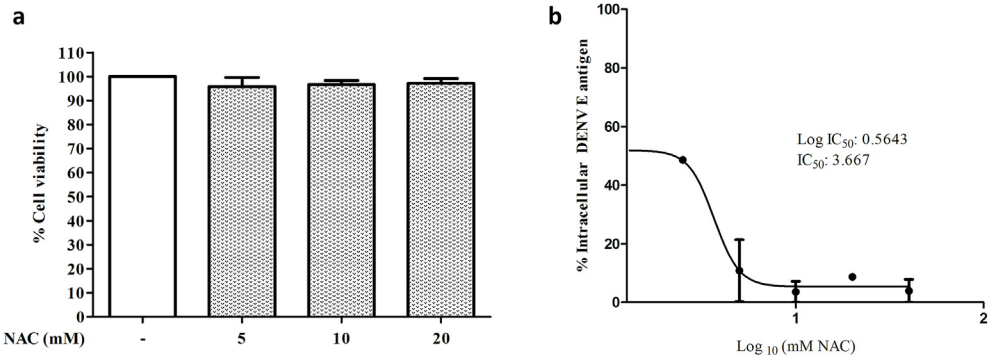

Fig. 1. Cytotoxicity assay and IC_{50} of NAC. HepG2 cells were treated with NAC at concentrations of 5 mM, 10 mM, or 20 mM. (A) Cell viability was determined. (B) NAC was serially diluted and treated with the DENV-infected HepG2 cells. The percentage intracellular DENV E antigen in the NAC treated groups and untreated control group was determined. The concentration that reduced the 50% intracellular DENV E antigen was calculated and is referred as IC_{50} value.

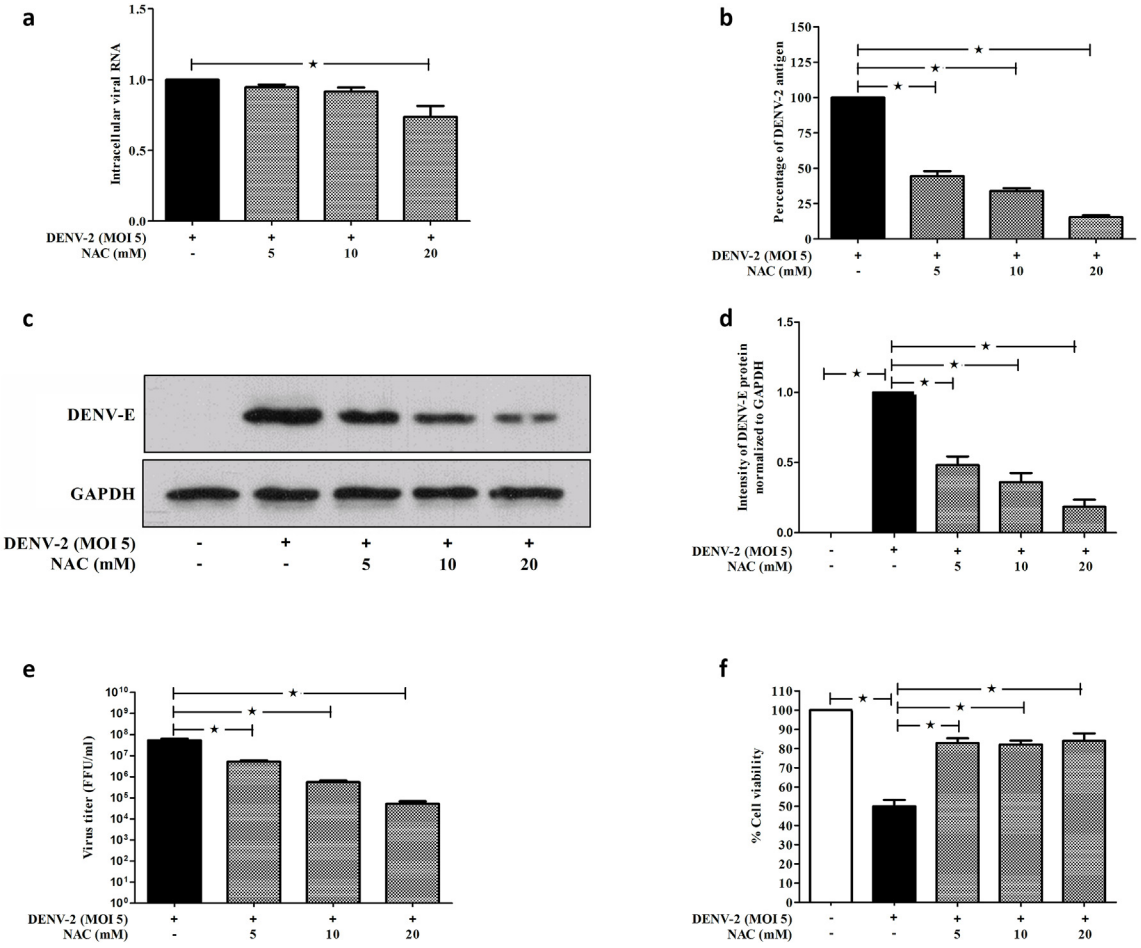

Fig. 2. NAC impeded different stages of DENV life cycle. HepG2 cells were infected with DENV-2 at an MOI of 5, after which they were treated with vehicle or NAC at concentrations of 5 mM, 10 mM, or 20 mM. Cell viability and DENV E expression at different steps of the DENV life cycle were analyzed. The results are described, as follows: (A) Intracellular viral RNA (B) Intracellular viral protein synthesis; (C) Western blot analysis of DENV E protein expression; (D) Densitometry analysis of DENV E expression (E) Extracellular viral product after exocytosis; (F) Cell viability analysis.

mice upregulated the expressions of pattern recognition receptors (PRRs), including Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Fig. 6A). Similarly, the expressions of type I interferons (IFN) including IFN-α and IFN-β, and its receptor IFNAR1 were significantly upregulated in DENV-infected HepG2 cells when treated with NAC (Fig. 6B). The interferon-stimulated genes (ISG) including OAS-1 and OAS-2 were also found to be significantly upregulated in DENV-infected HepG2 cells when treated with NAC (Fig. 6C). Our results hints NAC treatment reduced DENV infection in HepG2 cells by the upregulation of host antiviral response.

3.5. NAC treatment improved clinical manifestations in DENV-infected mice

DENV-infected mice experienced significant weight loss compared to the weight status of uninfected control mice (Supplementary Fig. 1A). Interestingly, NAC treatment in DENV-infected mice had the effect of restoring their lost weight compared to that of DENV-infected control mice over the course of the experiment schedule (Supplementary Fig. 1A). White blood cell (WBC) and platelet (PLT) counts dropped in DENV-infected mice (Fig. 7A and B), which suggests that DENV infection in mice effectuated the onset of leucopenia and thrombocytopenia. NAC treatment reversed these clinical symptoms in DENV-infected mice (Fig. 7A and B), suggesting NAC as an efficient modulator of leucopenia and thrombocytopenia in DENV-infected mice.

3.6. NAC treatment reduced liver injury in DENV-infected mice

Serum levels of liver transaminases, including ALT and AST, were estimated to characterize liver injury. DENV-infected mice showed elevation of both AST and ALT (Fig. 8A and B). In addition, and interestingly, both of these parameters were reduced in DENV-infected mice treated with NAC (Fig. 8A and B). The DENV-infected mice displayed classical signs of liver injury (Fig. 8C) including the presence of inflammatory cells and ballooning of the hepatocytes. Extensive tumefaction was observed in the hepatocytes of DENV-infected mice. Loosening of the sinusoid spaces, Kupffer cell hyperplasia and cellular necrosis were the other signs of liver injury observed in the DENV-infected mice. DENV-infected mice treated with NAC exhibited a lesser degree of hepatocyte ballooning. The sinusoids were rigid with lesser cell death when DENV-infected mice were treated with NAC (Fig. 8C). Additionally, the livers of DENV-infected mice weighed more than the livers of uninfected controls and interestingly, NAC treatment in DENV-infected mice reduced liver tissue weight (Supplementary Fig. 1B). A significantly higher cleaved caspase-3 expression was observed in DENV-infected mice and NAC treatment reduced this expression (Fig. 8D and E), which suggests its efficacy for suppressing DENV-induced liver cell apoptosis.

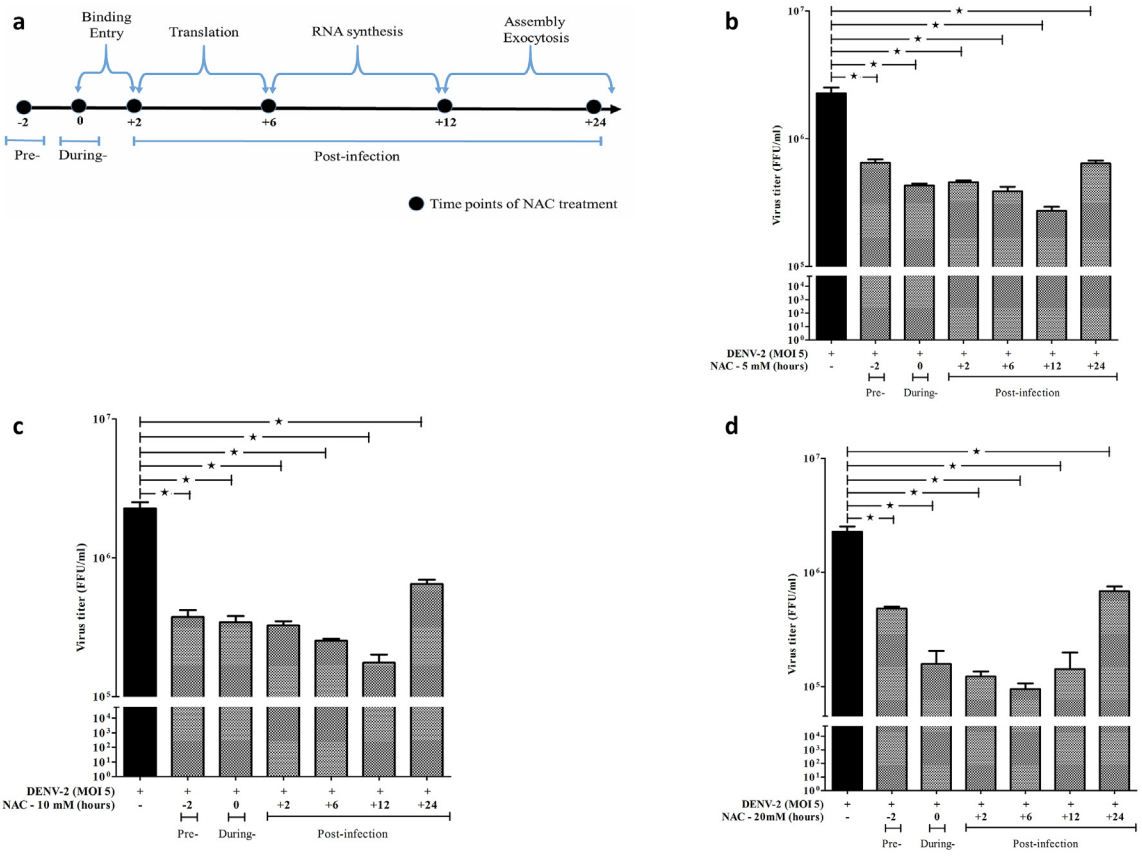

Fig. 3. Time of addition assay with different concentrations of NAC. HepG2 cells were infected with DENV-2 at an MOI of 5 and were treated with 5 mM, 10 mM and 20 mM concentrations of NAC at different point of time including pre-, during- and post-infection (−2, 0, +2, +4, +6, +12, +24 h). Infectious virion production was analyzed using FFU assay. (A) Graphical representation of the experimental design is shown and the results were represented as (B) time of addition with 5 mM NAC (C) time of addition with 10 mM NAC and (D) time of addition with 20 mM NAC.

3.7. NAC treatment suppressed virus replication in the livers of DENV-infected mice

Our results revealed a greater than one-fold reduction in DENV-NS1 viral RNA in the livers of DENV-infected mice treated with NAC (Fig. 9A). Similarly, DENV-infected mice that were treated with NAC showed a significant reduction in the viral FFU in the liver homogenates (Fig. 9B). Western blot analysis was then conducted to estimate DENV E protein expression in liver samples. DENV-infected mice treated with NAC showed a significant reduction in the expression of DENV-E protein (Fig. 9C and D).

Viral NS1 copies in the serum samples collected on day 3 and 7 post-infection was quantified. DENV-infected mice that were treated with NAC showed a significant reduction in the viral NS1 copies in the serum on both day 3 and day 7 post-infection (Fig. 9E); however, the viremia was found to be subsiding on day 7 compared to that of day 3 post-infection (Fig. 9E).

3.8. NAC reduced the oxidative damage in the livers of DENV-infected mice

DENV-induced liver injury in mice was found to be associated with reduced SOD (Fig. 9E) and CAT (Fig. 9F) activities; however, NAC treatment reversed these effects (Fig. 9E and F) to restore and maintain redox balance. These results suggest that treatment with NAC in DENV-infected mice is able to maintain antioxidant enzymes in the liver.

3.9. NAC modulated the phosphorylation of JNK in the livers of DENV-infected mice

Proteins extracted from liver tissues were used to study the

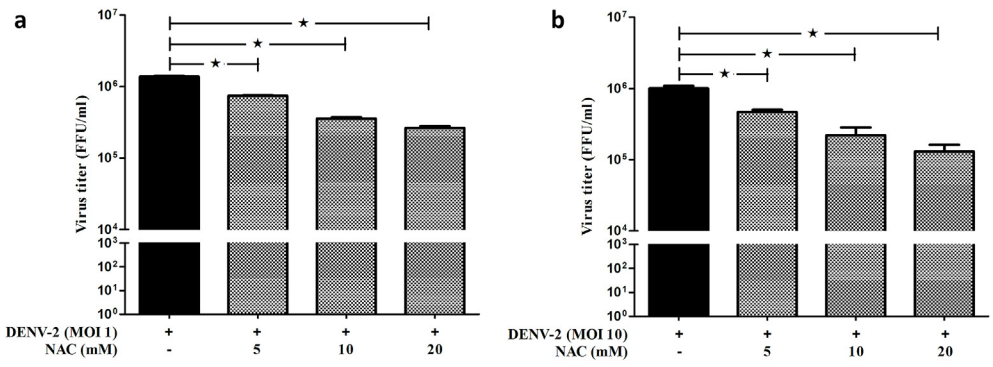

Fig. 4. Effect of NAC on different MOI of DENV infection. HepG2 cells were infected with DENV-2 at different MOI including 1 and 10, after which they were treated with vehicle or NAC at concentrations of 5 mM, 10 mM, or 20 mM. Infectious virion production was analyzed using FFU assay and is represented as (A) MOI-1 and (B) MOI-10.

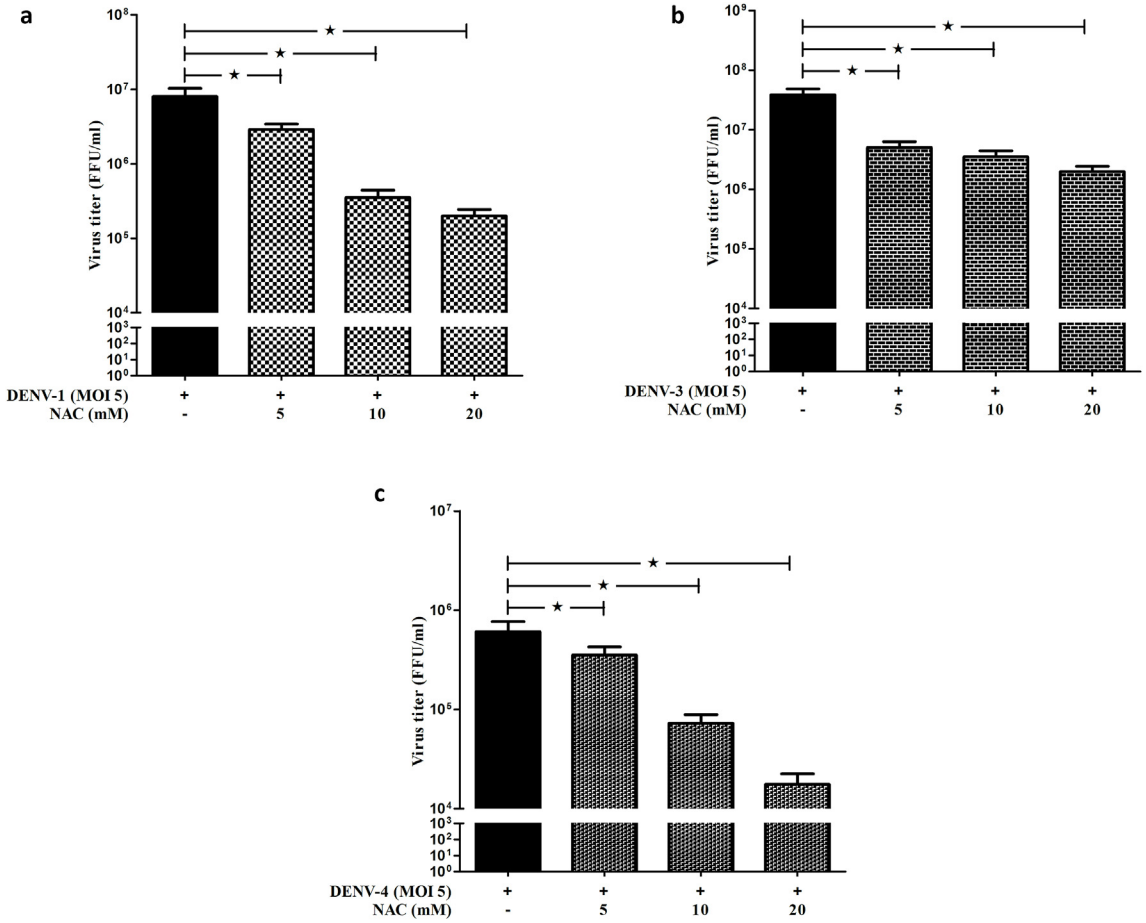

Fig. 5. Effect of NAC on different serotypes of DENV infection. HepG2 cells were infected with DENV-1 or DENV-3 or DENV-4 at a MOI of 5, after which they were treated with vehicle or NAC at concentrations of 5 mM, 10 mM, or 20 mM. Infectious virion production was analyzed using FFU assay and is represented as (A) DENV-1 and (B) DENV-3 and (C) DENV-4.

phosphorylation of JNK1/2. The phosphorylation of JNK1/2 was much higher in DENV-infected mice; however, a reduction in phosphorylation was observed in DENV-infected mice treated with NAC (Fig. 10A and B).

3.10. NAC exerted antiviral responses in the liver of DENV-infected mice

To further understand how NAC treatment reduces virus replication in the liver of DENV-infected mice, a pathway-specific microarray screening of 84 antiviral genes was performed. An additional DENV-infected group of mice treated with a JNK inhibitor (SP600125) was also introduced into the experiments. DENV replication in this newly introduced group was not found to be reduced, when compared to that of infected controls. We hypothesized that comparing the antiviral gene expression profiles of both the NAC-treated and SP600125-treated groups of mice would help to differentiate the antiviral responses compared to that of infected control group. The results were normalized with the expression of GAPDH, and the fold increase or fold decrease of genes that were found to be highly significant are represented in the Table 4.

Real-time polymerase chain reaction (RT-PCR) analysis was performed for the significant antiviral genes, with the use of specific primer sets to validate the microarray analysis. DENV-infected mice that had liver injury demonstrated upregulated expressions of PRRs, including RIG-I, MDA5 and mitochondrial antiviral signaling protein (MAVS) (Fig. 11A). Interestingly, NAC treatment in DENV-infected mice upregulated these antiviral genes (Fig. 11A); however, no such upregulation was observed in the SP600125-treated mice. Toll-like receptor (TLR) (i.e., TLR-3) expression was also higher in the livers of DENV-infected mice; however, this expression was much higher in DENV-infected mice treated with NAC (Fig. 11A). Interestingly, DENV-infected mice treated with SP600125 showed no upregulation in the expression of TLR-3 (Fig. 11A). Upregulated expressions of IFN signaling genes, including IRF-7, IFNA2, IFNB1 and IFNAR1 were observed in DENV-infected group of mice; however, these genes were highly upregulated in DENV-infected mice treated with NAC (Fig. 11B). DENV-infected mice treated with SP600125 also showed upregulation in IFNA2 and IFNB1 (Fig. 11B), but the levels of upregulation were found to be lower than those observed in DENV-infected mice treated with NAC. Interestingly, no significant upregulation of the IFN-receptor (IFNAR1) or IRF-7 was observed in DENV-infected mice treated with SP600125 (Fig. 11B). The interferon-stimulated gene OAS-2 was also found to be upregulated in the livers of DENV-infected mice and NAC increased its expression compared to the expression observed in DENV-infected control mice (Fig. 11C). These results suggest that NAC treatment is able to influence the antiviral signaling mechanism (mainly the type-1 IFN responses) in DENV-infected mice, which restricts virus replication to reduce DENV-induced liver injury. A schematic representation on the effect of NAC in the expressions of antiviral genes is shown in Fig. 12.

4. Discussion

In the present study, we conducted experiments in hepatoma cell line (HepG2 cells), in which DENV replication and apoptosis (Thepparit et al., 2004; Thongtan et al., 2004) were observed. NAC was previously

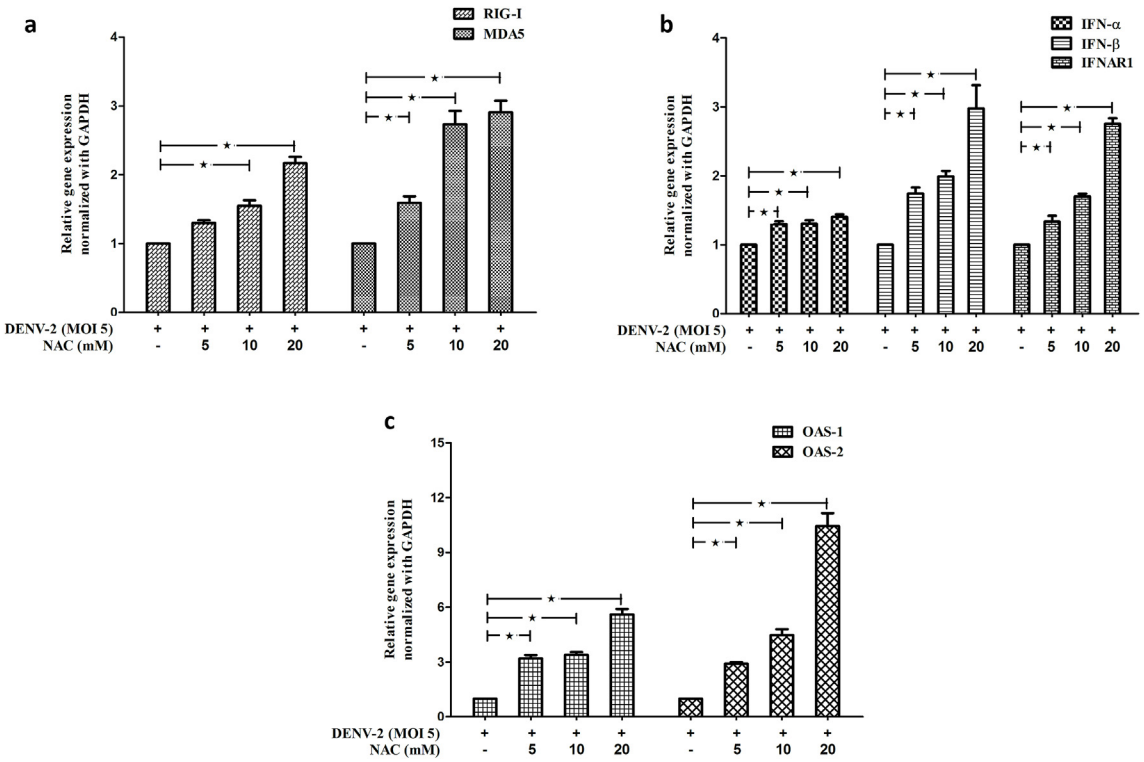

Fig. 6. NAC treatment exerted antiviral gene responses in the DENV-infected HepG2 cells. HepG2 cells were infected with DENV-2 at an MOI of 5, after which they were treated with vehicle or NAC at concentrations of 5 mM, 10 mM, or 20 mM. RNA samples were prepared from the cell lysates and converted to cDNA. RT-PCR was conducted, and the Ct values were normalized to that of GAPDH. The mRNA expressions of (A) PRRs including RIG-I; and MDA-5; (B) IFN responses including IFN-α; IFN-β; and IFNAR1; and, (C) IFN effector gene, OAS-1; and, OAS-2 is shown.

shown to inhibit the replication of respiratory syncytial virus (RSV) (Mata et al., 2011) and our results were similar in DENV-infected HepG2 cells. The NAC dosages used did not interfere the cell viability in HepG2 cells. The concentration of NAC to reduce 50% of the infectivity was determined in DENV-HepG2 cells, which is comparable to that of the minimum concentration of NAC used to treat DENV-infected HepG2 cells. Specifically, we used the dosages of NAC including 5 mM, 10 mM and 20 mM in the current study to treat DENV-infected HepG2 cells and optimized all the experiments with these doses and interestingly, those doses were able to effectively reduce infectious virion production.

The present study investigated the effect of NAC on different stages of DENV life cycle. During the life cycle of flaviviruses, the genomic RNA were translated to viral proteins in 6 h post-infection and viral RNA synthesis happens after 6 h post-infection; and the viral progeny were assembled and undergone exocytosis after 12 h post-infection (Chambers et al., 1990; Qing et al., 2009; Wang et al., 2015). Previously, time of addition of drugs were conducted with DENV-infected cell lines (Diosa-Toro et al., 2019; Kato et al., 2016) and our finding suggests NAC treatment obstructed different stages of DENV replication in HepG2 cells and further, the time of addition assay steadily supported these findings. Our finding suggests significant effect of NAC in the DENV protein synthesis; however, the reduction of intracellular viral RNA level seems to be less effective. Previously, a natural product library screening identified Narasin for its anti-DENV activity by reducing the DENV protein expression, but not the viral RNA expression (Low et al., 2011). Even though the dosage, 20 mM of NAC shows significant reduction in the viral RNA, more studies are necessary to conclude the effect of NAC in the viral RNA synthesis step. The

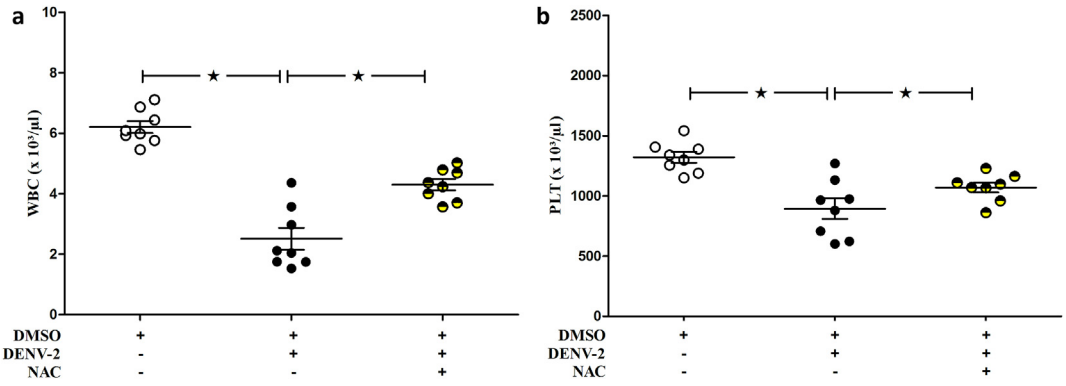

Fig. 7. NAC treatment improved the clinical hematology of DENV-infected mice. BALB/c mice were infected with DENV-2 (4×10^5 FFU/ml), and then treated with NAC (150 mg/kg) dissolved in 2% DMSO. DENV-infected 2% DMSO-treated and mock-infected 2% DMSO-treated groups of mice were used as positive and negative controls, respectively. Blood samples were collected on day 7 post-infection for analysis of clinical hematology. White blood cell (WBC) count (A) and platelet (PLT) count (B) are shown.

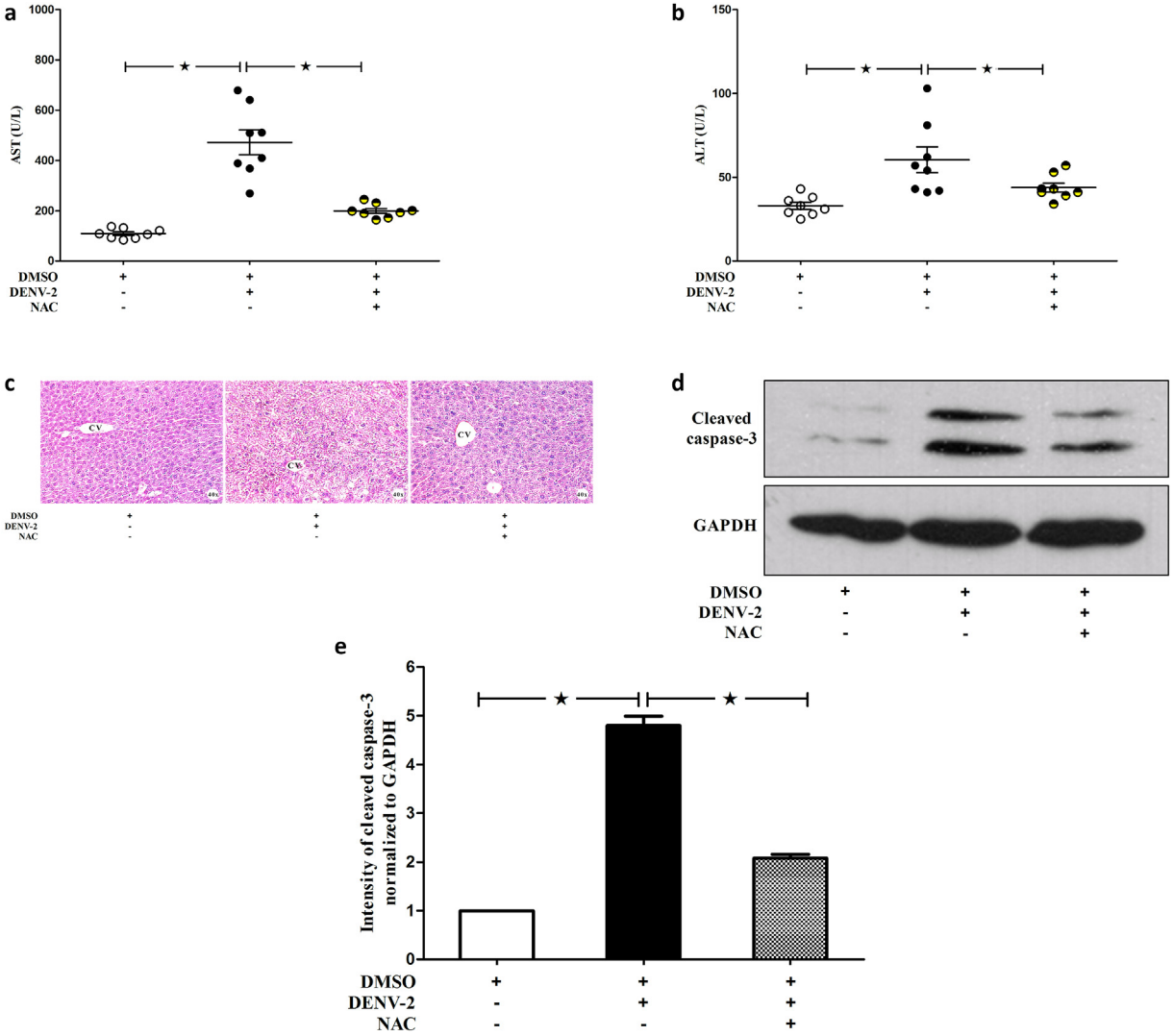

Fig. 8. NAC treatment reduced liver injury in DENV-infected mice. BALB/c mice were infected with DENV-2 (4×10^5 FFU/ml), and then treated with NAC (150 mg/kg) dissolved in 2% DMSO. DENV-infected 2% DMSO-treated and mock-infected 2% DMSO-treated groups of mice were used as positive and negative controls, respectively. Blood samples were collected on day 7 post-infection, and serum was prepared for the estimation of liver enzymes. Liver tissues were excised after sacrifice on day 7. Histopathologic analysis with H&E staining was then conducted. Western blot analysis was performed on protein samples obtained from liver homogenates, and the blots obtained were quantitated by ImageJ densitometry analysis. The results are shown, as follows: (A) AST; (B) ALT; (C) Histopathologic analysis with H&E staining; (D) Western blot analysis of cleaved caspase-3 expression and (E) Densitometry of cleaved caspase-3 expression.

upregulation in the expressions of antiviral genes including the PRRs, IFNs and ISGs was evident with the reduction in DENV production; suggesting the possible antiviral mechanism in the DENV-infected HepG2 cells, when treated with NAC. The reduction in the infectious virion production was found to be consistent when NAC at dosages 5 mM, 10 mM and 20 mM was treated with DENV-infected HepG2 cells at different MOI including 1, 5 and 10. In addition, NAC treatment in the HepG2 cells infected with different serotypes of DENV at MOI-5 also exhibited reduction in the infectious virion production.

Further, to better understand, we set forth to investigate the antiviral effect of NAC in an animal model of DENV-induced liver injury, in which DENV replication was evident (Sreekanth et al., 2014). Leucopenia, thrombocytopenia, and elevation of liver transaminases were observed in DENV-infected mice, and these clinical responses were similarly observed in DENV-infected humans (Azin et al., 2012; Binh et al., 2009). In DENV-infected mice, serum transaminases levels were at the peak on day 7 with typical signs of liver injury (Franca et al., 2010; Paes et al., 2005); and these findings were evident in our previous studies (Sreekanth et al., 2014, 2017); however, a higher viremia was observed in the serum on day 3 compared to that of day 7. Intravenous was administration of NAC at a dosage of 150 mg/kg was reported to be safe and effective in patients with DENV-induced acute liver failure (Kumarasena et al., 2010). The 150 mg/kg dose of NAC used in the present study is consistent with the dose that moderated liver injury in mice with hepatic ischemia-reperfusion injury (Lee et al., 2012). Importantly, the dosage of NAC given to DENV-infected mice in the present study alleviated leucopenia, thrombocytopenia, and liver transaminases. The histopathologic result supports the efficacy of NAC for reducing liver injury in DENV-infected mice. Increased expression of cleaved caspase-3 was observed in both *in vitro* cultures (Liao et al., 2010) and animal models of DENV-induced liver injury (Sreekanth et al., 2014), which suggests hepatic cell apoptosis as one of the important causes of liver injury during DENV infection. In the present study, DENV-infected mice treated with NAC showed reduced expression of cleaved caspase-3 compared to DENV-infected control mice, which suggests the efficacy NAC for suppressing hepatic cell apoptosis in DENV-infected mice. These findings are similar to those observed after MAPK inhibitors were used to treat DENV-infected mice that exhibited liver injury (Sreekanth et al., 2014, 2016, 2017). Our findings suggest the therapeutic efficacy of NAC for moderating DENV-

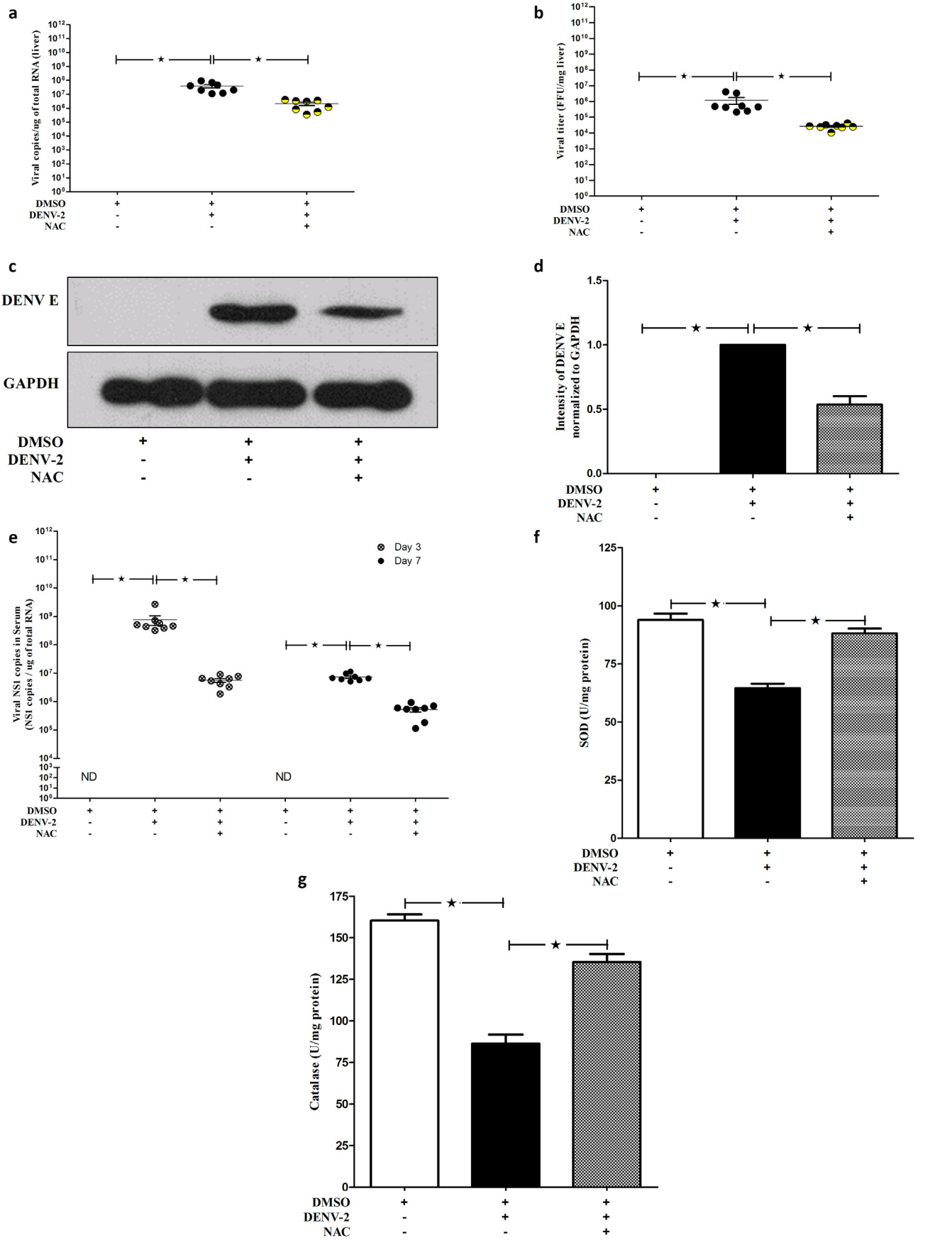

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Fig. 9. NAC treatment reduced virus replication and maintained the antioxidant enzymes in DENV-infected mice. BALB/c mice were infected with DENV-2 (4×10^5 FFU/ml), and then treated with NAC (150 mg/kg) dissolved in 2% DMSO. DENV-infected 2% DMSO-treated and mock-infected 2% DMSO-treated groups of mice were used as positive and negative controls, respectively. DENV NS1 viral RNA in liver tissue and serum was quantified using an *in vitro*-derived viral standard (standard curve method). A FFU assay was conducted with liver homogenates fixed in RPMI media. Western blot analysis was conducted on protein samples obtained from liver homogenates, and the blots obtained were quantitated by ImageJ densitometry analysis. For the antioxidant enzyme (SOD and catalase) activity assay, protein samples were prepared from liver homogenates using standard protocols. SOD and catalase absorbance was read at 450 nm and 570 nm, respectively. The results are given, as follows: (A) DENV NS1 viral RNA quantification in the liver; (B) FFU assay from liver homogenates; (C) Western blot analysis of DENV E expression (D) Densitometry analysis of DENV E expression (E) DENV NS1 viral RNA quantification in the serum (F) SOD activity and (G) Catalase activity.

associated clinical manifestations and liver injury; however, randomized trial is necessary to determine the appropriate dosage, efficacy, and safety in human DENV infection.

The results of liver tissue virus quantification experiments were consistent with our observations from DENV-infected HepG2 cells. NAC treatment in influenza virus-infected mice inhibited virus replication and ameliorated virus-induced lung injury (Zhang et al., 2014a). Our finding portends the therapeutic prospect of NAC to restrict virus replication in DENV-infected patients, and suggests further investigations into the effect of NAC on other endemic zoonotic viruses.

Oxidative stress in DENV-infected patients was previously correlated with thrombocytopenia (Soundravally et al., 2008). In DENV-infected dendritic cells, intracellular ROS was reported to be vital to determine the level of apoptosis (Olagnier et al., 2014). Antioxidant enzymes, including SOD and CAT, were reported to have significant effect in DENV-infected patients (Gil et al., 2004; Valero et al., 2013). In DENV-infected mice, these antioxidant enzymes were reported to be reduced, and resulted in elevated cytokines, including TNF-α and interleukin-6 (Wang et al., 2013). Consistent with previous findings, we observed significant reduction in both SOD and CAT in DENV-infected mice, and that NAC treatment was able to maintain these antioxidant enzymes. In porcine circovirus-infected PK15 cells NAC treatment showed significant reduction in the oxidative stress and inhibition of virus replication (Chen et al., 2012). Our findings need to be further elucidated to establish whether or not these antioxidant enzymes directly influenced the suppression of DENV replication.

The JNK inhibitor, SP600125 reduced DENV replication in macrophages (Ceballos-Olvera et al., 2010); but, not in DENV-infected mice (Sreekanth et al., 2017). In the present study, NAC treatment in DENV-infected mice reduced JNK1/2 phosphorylation and suppressed DENV replication in the liver. As such, our findings suggest NAC as a promising modulator of DENV-induced liver injury via inhibition of virus replication, as well as by regulating JNK phosphorylation.

In Japanese encephalitis virus (JEV) infection, several antioxidants including NAC, were reported to have antiviral activity (Zhang et al., 2014b). Our findings in the DENV-infected HepG2 cells were found to be consistent in the NAC treated DENV-infected mice. The host innate immune system detects the structural patterns of viruses by their pathogen-associated molecular patterns (PAMPs), and they are recognized by pattern recognition receptors (PRRs). RIG-1 and MDA-5 were identified as being involved in viral dsRNA recognition during innate immunity (Loo et al., 2008), and they were found to be important for the production of interferons (IFNs) (Kato et al., 2006). RIG-I-like receptors (RLRs) then activate the MAVS in the mitochondrial and peroxisome membranes that regulate the post-translational modifications of RIG-I and MDA5 for type-1 IFN production (Takeuchi and Akira, 2010). In DENV-infected cells, RIG-I, MDA5, and TLR-3 expressions were

Table 4
The antiviral gene expression profiles of DMSO treated DENV-infected mice, DENV-infected mice treated with NAC or SP600125, normalized to mock-infected DMSO treated mice.

Gene	Gene description	mRNA expression			
IL18	Interleukin 18	1	53.5905	8.3660	24.5285
CXCL10	IP-10	1	22.9727	2.6507	8.551
IL6	Interleukin 6	1	12.0849	5.2311	6.2226
CCL5	RANTES	1	12.0738	1.8888	3.4822
IFNB1	Interferon beta	1	9.0849	17.2311	11.2226
CASP8	Caspase 8	1	8.7526	1.1567	2.6643
IFNA2	Interferon alpha 2	1	6.2535	13.9497	8.5661
CD40	Cd154	1	5.7371	1.4353	2.5157
MAPK8	JNK 1	1	4.8293	1.6425	1.2404
PYCARD	Apoptosis-associated speck-like protein containing a CARD	1	4.5692	1.4897	2.3251
CTSB	Cathepsin B	1	4.0867	1.1173	2.8284
DDX58	RIG-1	1	3.4147	8.2249	3.5373
TLR9	Toll like receptor 9	1	3.2308	4.8311	3.6268
IFNAR1	Interferon-alpha/beta receptor alpha chain	1	3.1487	7.3660	4.1435
TLR3	Toll like receptor 3 or CD288	1	3.1090	4.9040	2.6507
IFIH1	MDA5 (Melanoma Differentiation Associated protein 5)	1	3.0825	5.8819	3.2349
MAVS	Mitochondrial antiviral signaling protein	1	3.0620	3.9281	2.8801
OAS2	2′-5′-oligoadenylate synthetase 2	1	2.9395	5.5864	2.6974
IRF7	Interferon regulatory factor 7	1	2.3950	5.9053	2.2815
TLR7	Toll like receptor 7	1	1.4834	2.6133	1.3664
	DMSO	+	+	+	+
	DENV-2	−	+	+	+
	NAC	−	−	+	−
	SP600125	−	−	−	+

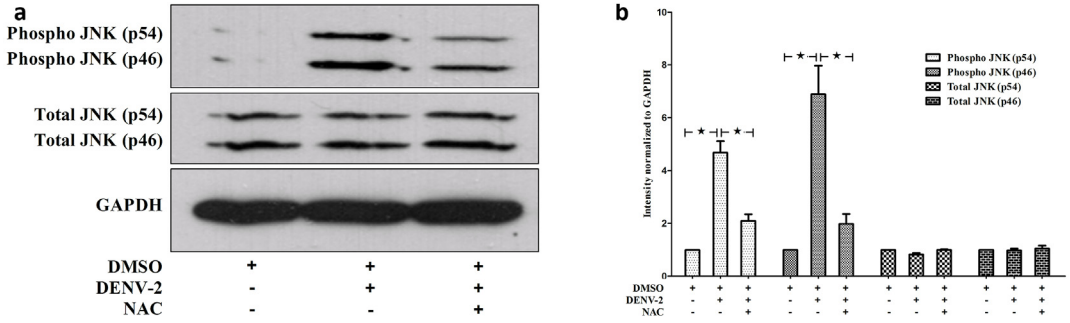

Fig. 10. NAC reduced oxidative stress and JNK phosphorylation in the livers of DENV-infected mice. BALB/c mice were infected with DENV-2 (4×10^5 FFU/ml), and then treated with NAC (150 mg/kg) dissolved in 2% DMSO. DENV-infected 2% DMSO-treated and mock-infected 2% DMSO-treated groups of mice were used as positive and negative controls, respectively. Protein samples were prepared from liver tissues, and then Western blot analysis was performed with normalization to that of GAPDH. Densitometry analysis of blots was conducted using ImageJ software normalized to individual GAPDH. (A) Western blot analysis of phosphorylated and total JNK expressions and (B) Densitometry analysis of phosphorylated and total JNK expressions.

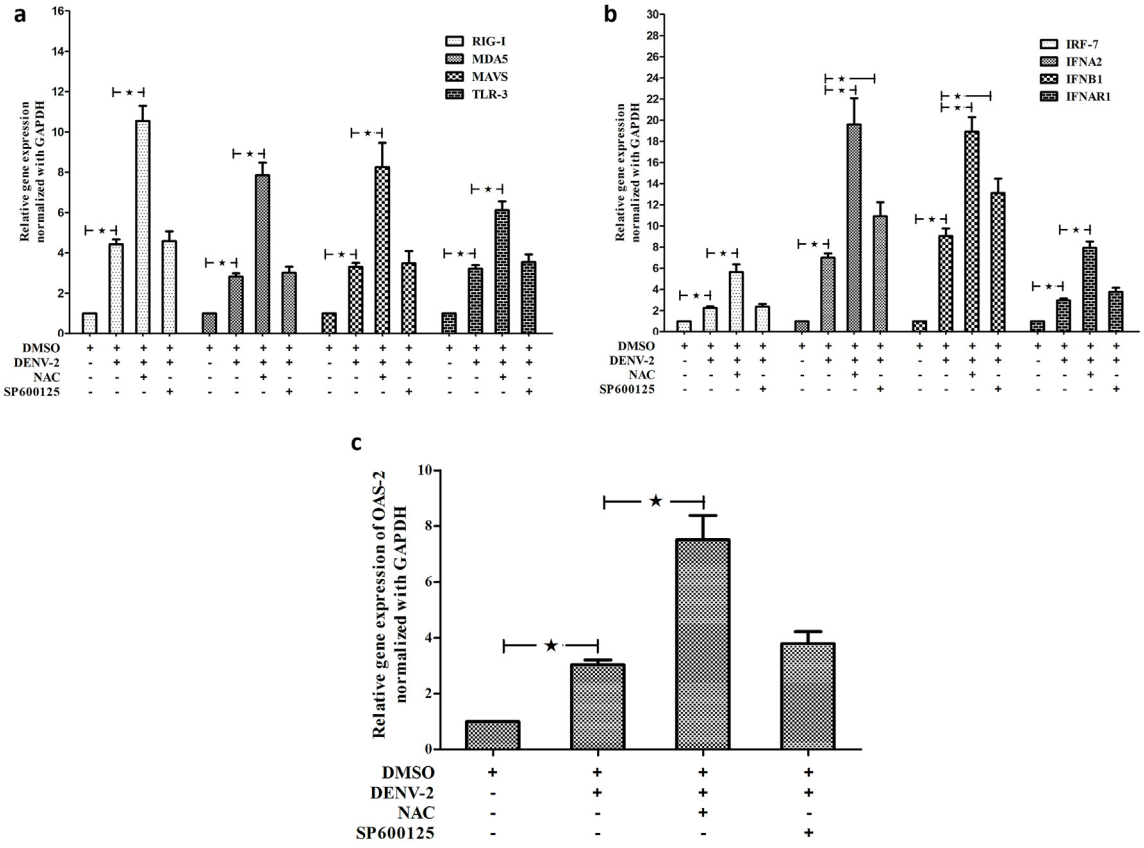

Fig. 11. NAC treatment exerted antiviral gene responses in the livers of DENV-infected mice. BALB/c mice were infected with DENV-2 (4×10^5 FFU/ml), and then treated with NAC (150 mg/kg) dissolved in 2% DMSO. DENV-infected 2% DMSO-treated and mock-infected 2% DMSO-treated groups of mice were used as positive and negative controls, respectively. Another group of DENV-infected mice that was treated with SP600125 was used as control group exhibiting no reduction in DENV replication. RNA samples prepared from liver tissues were converted to cDNA. RT-PCR was conducted, and the Ct values were normalized to that of GAPDH. The mRNA expressions of (A) PRRs including RIG-I; MDA-5; MAVS; and TLR-3; (B) IFN responses including IRF-7; IFNA2; IFNB1; and IFNAR1; and, (C) IFN effector gene, OAS-2 is shown.

upregulated to inhibit DENV replication (Nasirudeen et al., 2011). NAC treatment in DENV-infected mice effectuated a higher antiviral response of RIG-I, MDA-5 and MAVS compared to the responses observed in the infected controls and the SP600125-treated mice, which is evidence that is suggestive of reduced virus replication. TLRs mediates strong IFN responses during DENV infection; that restrict virus replication, resulting lower cytopathic effects (Tsai et al., 2009). In DENV-infected HepG2 cells, activation of TLR-3 blocked virus replication via IFN-β response (Liang et al., 2011), and that same activation was observed in DENV-infected mice when they were treated with NAC.

Interferons, including IFN-α, IFN-β, and IFN-γ, were previously described to restrict viral replication during DENV infection (Kanlaya et al., 2010; Sadler and Williams, 2008). Our findings suggest that the antiviral activity of NAC induces IFNs, including IFNA2, IFNB1, IRF-7, and the receptor IFNAR1; however, SP600125 failed to induce IFNAR1

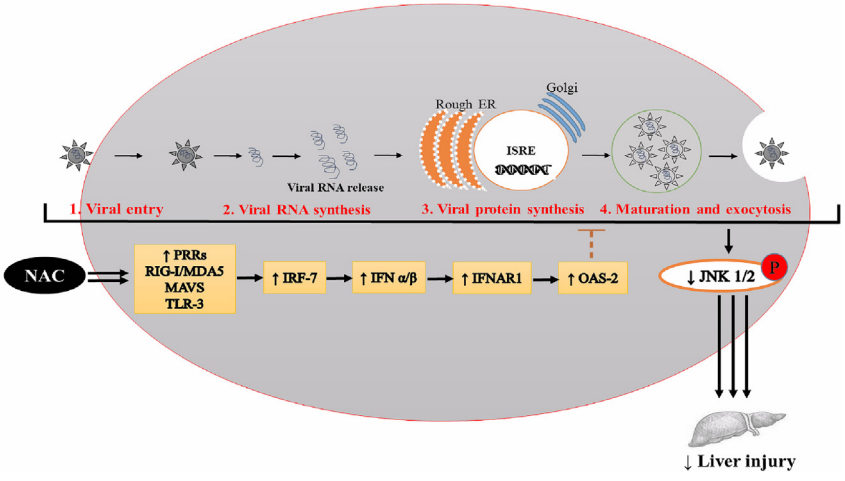

Fig. 12. Flow diagram describing the proposed mechanism of NAC in DENV-induced liver injury.

and IRF-7 expression. This may be one of the reasons that virus replication was not reduced when DENV-infected mice were treated with SP600125 (Sreekanth et al., 2017). Mice lacking IFNAR1 develop severe DENV infection, and the protective role of IRF-7 was previously reported (Carlin et al., 2017). IRF-7 plays a more influential role in initiating IFN-α/β response than IRF-3; however, the combined effects of IRF-7 and IRF-3 are even more efficient than the role of IRF-3 alone (Chen et al., 2013). In DENV-infected macrophages, SP600125 was reported to inhibit DENV replication (Ceballos-Olvera et al., 2010), though the interferon responses were not investigated to compare with our observations in DENV-infected mice treated with SP600125 (Sreekanth et al., 2017).

Type-1 IFN responses initiate the activation of IFN-stimulated genes, including the 2′,5′-oligoadenylate synthetase (OAS) family, that establishes a steady antiviral state against viruses and they are referred to as antiviral effector molecules. OAS-1 and OAS-3 showed anti-DENV activity (Simon-Loriere et al., 2015), and recently, minocycline treatment was reported to upregulate the expression of OAS-1 and OAS-3 to limit DENV replication in HepG2 cells (Leela et al., 2016). DENV-infection results in the early induction of OAS1; however, OAS2 and OAS3 were upregulated at the later stages of DENV replication (Bordignon et al., 2008; Warke et al., 2003). In DENV-infected A549 cells, over expressions of OAS1 and OAS3 were able to limit DENV replication; however, expression of OAS-2 was not efficacious for reducing DENV replication (Lin et al., 2009). The role of interferon-stimulated gene OAS2 was not previously defined in the host defense mechanism of DENV-infected mice. We report a three-fold upregulation in the expression of OAS-2 in DENV-infected mice. Importantly, NAC treatment upregulated this response in DENV-infected mice, which suggests the antiviral effect of NAC treatment involving the expression of OAS2.

5. Conclusions

NAC treatment demonstrated an ability to reduce virus production in both DENV-infected HepG2 cells and DENV-infected mice; which was likely to be mediated via the upregulation of antiviral interferon responses. Moreover, NAC treatment modulates DENV-induced oxidative stress and JNK1/2 phosphorylation in DENV-infected mice thereby reducing liver injury. Our findings suggest the novel therapeutic potential of NAC in DENV-induced liver injury and recommend evaluating its efficacy and safety in randomized trials of DENV infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.03.011.

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