ARTÍCULO DE REVISIÓN

Oxidative-nitrosative stress and dengue disease: a systematic review of *in vivo*/*in vitro* studies

Estrés oxidative-nitrosativo y dengue: revisión sistemática de estudios *in vivo* e *in vitro*

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ABSTRACT

Objective: In this systematic review the aim was to summarise the *in vivo*/*in vitro* evidence on the role of oxidative-nitrosative stress in pathogenesis of dengue.
Methods: We searched electronic databases (PubMed, EMBASE, The COCHRANE library, ScienceDirect, Scopus, SciELO, LILACS via Virtual Health Library, Google Scholar) using the term: dengue, dengue virus, severe dengue, oxidative stress, nitrosative stress, antioxidants, oxidants, free radicals, oxidized lipid products, lipid peroxides, nitric oxide, and nitric oxide synthase. Articles were selected for review by title and abstract excluding letter, review, epidemiological studies, and duplicates studies. Selected articles were reviewed for used animal model or cell cultures, original purposes, strain of virus or type of antibody, main outcomes, methods, and oxidative-nitrosative stress markers values.
Results: In total, 4330 non-duplicates articles were identified from computerized searches of reference databases, of which 32 were eligible for full text searching. The results of *in vivo* studies were obtained from monkey and knockout and/or wild-type mice. Human peripheral blood mononuclear cells were cell cultures most commonly used in identified *in vitro* studies, following by human endothelial cells cultures. DENV-2 strains were most used.
Conclusions: In conclusion, a large body of *in vivo* and *in vitro* evidences showed that oxidative/nitrosative stress can be related to production of pathogenesis-related protein, increased susceptibility of mice to DENV infection, hemorrhage

development in mice, proinflammatory cytokines and transcriptional factor expression, and DENV replication in various cell cultures.

Keywords: dengue; severe dengue; dengue virus; oxidative stress; nitrosative stress; biological markers; systematic review (source: MeSH).

RESUMEN

Objetivo: sistematizar las evidencias *in vivo*/*in vitro* de la participación del estrés oxidativo-nitrosativo en el curso de la infección por virus del dengue.
Métodos: revisión sistemática de estudios observacionales en las bases de datos (PubMed, EMBASE, The COCHRANE library, ScienceDirect, Scopus, SciELO, LILACS via Virtual Health Library, Google Scholar) utilizando las siguientes palabras clave: dengue, dengue virus, severe dengue, oxidative stress, nitrosative stress, antioxidants, oxidants, free radicals, oxidized lipid products, lipid peroxides, nitric oxide y nitric oxide synthase. La selección inicial fue realizada a partir del título y resumen excluyéndose: cartas para editor, revisiones, estudios con diseños epidemiológicos y estudios duplicados. A cada artículo seleccionado, se le revisó el objetivo o propósito, cultivos celulares o modelos animales utilizados, cepas víricas o tipo de anticuerpos utilizados, métodos y valores de los marcadores de estrés oxidativo-nitrosativo.
Resultados: de 4330 publicaciones encontradas, 32 estudios cumplieron con los criterios de inclusión. Se utilizaron primates no humanos y ratones knockout o tipo salvaje para la obtención de las evidencias *in vivo*. Los cultivos celulares más utilizados fueron de células mononucleares de sangre periférica y de células endoteliales humanas. Las cepas más utilizadas en los ensayos correspondieron al serotipo 2 del virus dengue.
Conclusiones: existen evidencias *in vivo*/*in vitro* que muestran la posible asociación entre el estrés oxidativo-nitrosativo con: producción de proteínas relacionadas con la patogénesis del dengue, incremento en la susceptibilidad de ratones por la infección por dengue, desarrollo de hemorragias en modelo de ratón, expresión de citoquinas proinflamatorias y replicación viral en varios cultivos de células tanto humanas como de origen animal.

Palabras clave: dengue; dengue grave; virus dengue; estrés oxidativo; marcadores biológicos; revisión sistemática (fuente: DeCS).

INTRODUCCIÓN

Dengue is a systemic viral disease with ubiquitous distribution in tropical and subtropical regions.[1] The transmission mechanism involves Aedes mosquito, being *A. aegypti* the main vector.[2,3]

The etiologic agent of this tropical disease is dengue virus (DENV), member of the family *Flaviviridae*, with four different antigenic serotypes (DENV-1 to -4). The DENV genome of plus strand RNA encodes three structural proteins (capsid, prM

and envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b y NS5).[4,5]

The productive infection *in vitro* has been proven in various cell types, such as dendritic cells, monocytes and macrophages, B and T lymphocytes, fibroblasts, endothelial cells, and hepatocytes. *In vivo*, monocytes and macrophages considered primary target cells.[6,7]

DENV infection can present a wide spectrum of clinical symptoms, and severity may vary according to age, ethnicity, genetic factors, immune status and underlying disease. It may also depend on the co-circulation of DENV serotypes and reinfection by different DENV serotypes.[8-12]

Moreover, it has been proposed the involvement of DENV infection-derived oxidative stress on the severity of dengue. This is based on their ability to trigger the release of proinflammatory cytokines, including TNF-alpha, participating in collective action in the immunopathogenesis of dengue diseas. [13]

By definition, oxidative stress is an imbalance between pro-oxidants and antioxidants in favour of the pro-oxidants.[14,15] Instead, nitrosative stress is defined as an indiscriminate nitrosilation of biological molecules.[16]

In the absence of an appropriate compensatory response from endogenous antioxidant defense system, the activation of several stress-sensitive intracellular signaling pathways have been reported. This activation involves the production of gene products that can lead to cell death and/or pathophysiological conditions.[16-19]

In biomedical research, *in vivo* and *in vitro* studies are reproducible system, more or less complex, that are used to study the cellular and molecular mechanisms that are involved in the physiopathology of diseases.[20]

For this reason, in this systematic review the aim was to summarise the *in vivo*/*in vitro* evidence on the role of oxidative-nitrosative stress in pathogenesis of dengue. This review is important because understanding the involvement of oxidative and nitrosative stress in dengue pathogenesis could have potential implications for prognosis and treatment.

METHODS

Our research protocol was based on the preferred reporting items of systematic review and meta-analysis (PRISMA) guidelines[21] and was registered on PROSPERO, an international database of prospectively registered systematic reviews in health and social care managed by Center for Review and Dissemination, University of York, on 13 November 2014; http://www.crd.york.ac.uk/PROSPERO (CRD42014014912).

We searched online databases (PubMed, EMBASE, The COCHRANE library, ScienceDirect, Scopus, SciELO, LILACS via Virtual Health Library, Google Scholar) for *in vivo* and *in vitro* studies that considered the role of oxidative and nitrosative stress in the pathogenesis of dengue. Selected articles were reviewed for used animal model or cell cultures, original purposes, strain of virus or type of antibody, main outcomes, methods, and oxidative stress markers values. In this systematic review, there is no restriction regarding to language or publication period.

Our search term included "dengue" and "dengue virus" combined with "oxidative stress", "nitrosative stress", "antioxidants", "oxidants", "free radicals", "oxidized lipid products", "lipid peroxides", "superoxide dismutase", "thioredoxin reductase", "nitric oxide", or "nitric oxide synthase". This electronic search strategy was supplemented by scanning the reference lists of all articles to identify additional studies that may have been missed during the initial search.

Articles were selected for review by title and abstract. Exclusion criteria were impossible extraction of data, no control group, dates from mosquito cells cultures, case reports, letter to the editor, review articles, proteomics and epidemiological studies. When multiple publications from the same study population were available, we included the most recent publication.

The systematic computerized literature search of published studies was carried out in December 2014.

Results and Discussion

Identification of studies

In total, 4330 non-duplicates articles were identified from electronic databases searches, of which 32 were eligible for full text searching (fig 1). Tables 1-2 present the citation, definitions and characteristics of each included *in vivo* and *in vitro* studies, respectively.

Table 1. *In vivo* evidences of oxidative and nitrosative stress involvement in dengue pathogenesis

Study First author, Year, (Reference)	Original purposes	Animal model	Virus	Outcomes
Misra A, 1996, (22)	To investigate the production of nitrite by the spleen cells of mice *in vitro* and *in vivo* following inoculation of DENV or CF	Swiss albino mice (6-8 weeks)	DENV-2, strain P23085	Maximum production of nitrite and peak value of cytotoxic activity occurred at day 11 of DENV inoculation.
Mukerjee R, 1996, (24)	To investigate whether CF2 induces production of nitrite in the spleen cells of mice	Swiss albino mice	DENV-2, strain P23085	The maximum production of nitrite at 60 minutes after the inoculation of CF2.
Misra A, 1996, (23)	To investigate the production of superoxide and peroxide and their role in the cytotoxic activity of CF2	Swiss albino mice (3-4 months)	DENV-2, strain P23085	The maximum release of peroxide occurred at 90 minutes after CF2 inoculation.
Yen YT, 2008, (27)	To investigate the molecular mechanism of dengue hemorrhage	C57BL/6 $iNOS^{-/-}$ mice	DENV-2, strain 16681	iNOS RNA transcripts were upregulated in tissues of

				hemorrhagic mice but not in mice injected with UV-inactivated DENV. $CD31^+$ vascular endothelial cells in hemorrhage tissues expressed both iNOS and nitrotyrosine beginning at days 2 p.i.
Garcia G, 2008, (40)	To determine behavior of NO in serum of *Macacus irus* inoculated with DENV-2 or sequential DENV-4/DENV-2 infection	*Macacus irus* (male, 4-6 kg)	DENV-2 strain A15 DENV-4 strain H241	High concentrations of NO were detected in monkeys with primary DENV-4 infection whereas those monkeys with sequential DENV-4/DENV-2 infection did not show NO concentrations over 100 μM.
Fagundes CT, 2011, (25)	To examine the role of IFN-γ, NO, IL-12 and IL-18 during dengue infection	C57BL/6J mice IFN-γ-deficient mice NOS2-deficient mice	DENV-2, strain P23085	In spleen of DENV-2-infected wild-type mice, NOS2 mRNA expression was significantly increased at 5 and 7 d.p.i. In liver of DENV-infected wild-type mice, NOS2 mRNA expression was significantly increased at 7 d.p.i. NOS2-deficient mice were markedly susceptible to DENV-2 infection. After DENV-2

				infection, viral loads in spleen were significantly greater in NOS2-deficient mice than wild type mice.
Costa VV, 2012, (26)	To characterize a novel model of DENV-3 infection in immunocompetent adult mice	C57BL/6J mice IFN-γ-deficient mice NOS2-deficient mice	DENV-3, strain JN697379	In spleen of DENV-3-infected wild-type mice, NOS2 mRNA expression was significantly increased at 5 and 7 d.p.i. In liver of DENV-infected wild-type mice, NOS2 mRNA expression was significantly increased at 7 d.p.i. After DENV-3 infection, nitric oxide was not secreted by dendritic cells. NOS2-deficient mice were markedly susceptible to DENV-3 infection. In DENV-3-infected $NOS^{-/-}$ mice, viraemia and viral load in spleen and liver were significantly higher in comparison to WT

				mice.
Wang J, 2013, (28)	To investigate the inhibitory effect of GSH on oxidative stress induced by DENV-2 infection	SCID mice (six week-old, female)	DENV-2, strain Tr1751	In serum and liver in the mice after DENV-2 infection, total SOD activity was significantly decreased in comparison to uninfected SCID mice. MDA levels in serum and organs of the DENV-2-infected mice were significantly higher in comparison to controls. Hepatic CAT activity showed a significant decreased when compared with the controls. GSSG/GSH ratio showed a marked decrease after DENV-2 infection. Viraemia had a positive correlation with MDA levels. After treatment with GSH, DENV-2 titers, MDA, IL-6, and TNF-α levels in the serum were significantly

				decreased in comparison to controls. After treatment with a precursor of GSH, DENV-2 titers in the liver were significantly decreased in comparison to controls.
de Souza, KP, 2013, (29)	To characterize the immunopathology and neurovirulence that occurs in dengue infected hosts	C57BL/6J mice (8-10 weeks of age) NOS2-deficient mice	DENV-1, strain BH4 DENV-2, strain Pi59 DENV-3, strain MG20 strain MG21 strain Pi76	Mortality rates were significantly different between DENV-3-infected WT and $NOS2^{-/-}$ mice. The presence of virus appears to correlate with increased NOS2 and cytokine expression in the brain of WT mice between the 7^{th} and 8^{th} d.p.i.

CF: cytotoxic factor; CF2: cytotoxic factor 2; iNOS/NOS2: inducible nitric oxide synthase; NO: nitric oxide; d.p.i.: days post-infection; WT: wild type; GSH: glutathione;
MDA: malondialdehyde; CAT: catalase; GSH: reduced glutathione; GSSG: gluthatione disulfide.

Table 2. *In vitro* evidences of oxidative and nitrosative stress involvement in dengue pathogenesis

Study First author, Year, (Reference)	Original purposes	Cell culture	Virus or type of antibody	Outcomes
Misra A, 1996, (22)	To investigate the production of nitrite by the spleen cells of mice *in vitro* and *in vivo* following inoculation of DENV or CF	Mouse spleen cells	DENV-2, strain P23085	Maximum production of nitrite was observed after 45 minutes of CF treatment and at 72 hours after DENV inoculation.
Mukerjee R, 1996, (24)	To investigate whether CF2 induces production of nitrite in the spleen cells of mice	Mouse spleen cells	DENV-2, strain P23085	The maximum production of nitrite at 60 minutes after the inoculation of CF2.
Misra A, 1996, (23)	To investigate the production of superoxide and peroxide and their role in the cytotoxic activity of CF2	Mouse spleen cells	DENV-2, strain P23085	The maximum release of superoxide and peroxide occurred at 45 minutes and 90 minutes after CF2 inoculation, respectively. SOD treatment inhibited the production of superoxide and abrogates of cytotoxic activity of CF2 in a dose-dependent manner while

				peroxide production was increased.
Khare M, 1997, (31)	To investigate the production of nitrite and their role in the transmission of dengue virus-induced suppressor signal	Murine peritoneal macrophages	DENV-2, strain P2385	Pretreatment of murine macrophages with anti-suppresor factor antiserum or arginase inhibited production of nitrite
Marianneau P, 1999, (32)	To investigate the ability of dengue virus to invade human primary Kupffer cells	Human primary Kupffer cells	DENV-1, strain Oster	80 % of Kupffer cells were labeled with anti-iNOS antibody at 1 hour after exposure to DENV. Increase in the concentratio n of nitrite or nitrate in the supernatants of DENV-infected Kupffer cells was constant.
Lin YL, 2000, (37)	To explore a correlation between DENV infection and up-regulated RANTES gene expression in liver cells	Chang liver cells	DENV-2, strain PL0146 DENV-3, strain 739079A	DENV-2 infection increased the GSSG/GSH ratio with infection time. All antioxidants used (NAC plus GSH, PDTC, or L-

				NAME) effectively suppressed RANTES mRNA expression.
Jan JT, 2000, (33)	To explore the apoptotic pathway in DEN-2 virus-infected human neuroblastoma cells	Human neuroblastoma cells	DENV-2, strain PL046	Level of intracellular superoxide anion was increased by 24 hours after DENV-2 infection, peaked at 48 h p.i., and at 72 h.p.i. decreased.
Lin CF, 2002, (37)	To explore the effects of antibodies against DENV NS1 on human endothelial cells and mouse vessel endothelium	Human microvascular endothelial cells	DENV-2, New Guinea C strain	After treatment with anti-NS1 IgG, the expression of iNOS, p53, and Bax were increased, whereas that the protein levels of Bcl-2 and Bcl-x_L decreased. Furthermore, the release of cytochrome c from

				mitochondria was observed. Treatment with the NOS inhibitor L-NAME inhibited the anti-NS1-mediated changes of p53, Bax, Bcl-2, and Bcl-x_L expression and cytochrome c release.

Overview of included in vivo and in vitro studies

Animal models. The results of *in vivo* studies were obtained from knockout and/or wild-type mice (Swiss albino mice, C57BL/6 inducible nitric oxide synthase-iNOS$^{-/-}$ mice, C57BL/6J IFN-g$^{-/-}$ mice, and severe combined immunodeficiency-SCID mice) or male monkeys (*Macacus irus*).

Cell cultures. Human peripheral blood mononuclear cells were cell cultures most commonly used in identified *in vitro* studies, following by human endothelial cells cultures.

DENV serotypes. In the most of the studies reviewed here, DENV-2 strains were used.

In vivo studies/Outcomes. Nitric oxide synthase 2-NOS2 RNA transcripts or mRNA expressions were evaluated in spleen, liver, brain of DENV-infected mice. Likewise, total superoxide dismutase-SOD activity, malondialdehyde-MDA levels, catalase-CAT activity, and oxidized glutathione-GSSG/reduced glutathione-GSH ratio were also determined in serum and organs of DENV-infected mice.

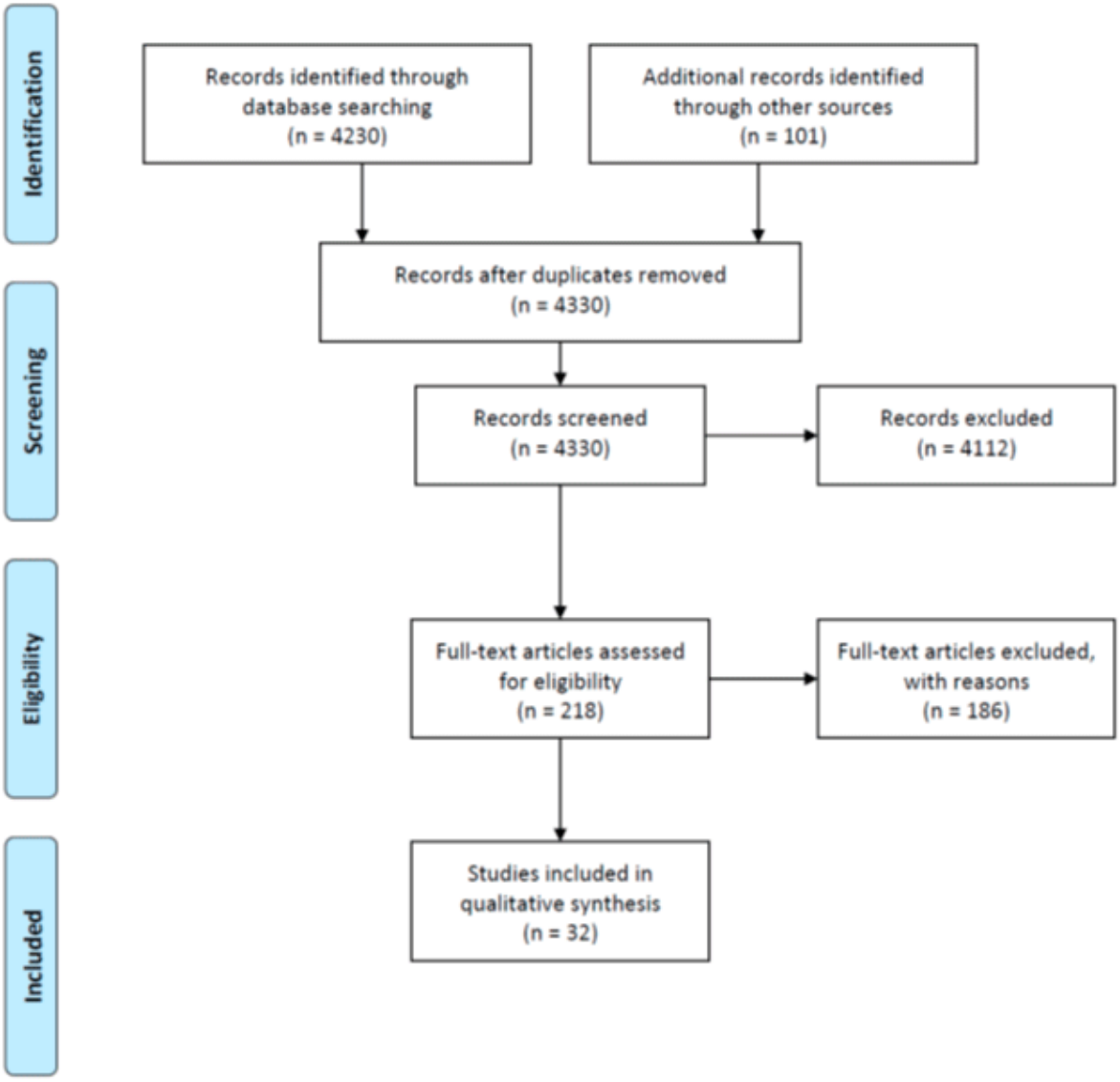

Fig. Flow diagram of studies included in the systematic review.

In vitro studies/Outcomes. NOS2 mRNA expressions were evaluated in human endothelial and mononuclear cell cultures. Total reactive oxygen species-ROS production was evaluated in dengue virus-infected human monocyte-derived dendritic cells and human hepatoma cells. In addition, MDA and GSH concentrations and CAT activity were determined in human mononuclear cell cultures. GSSG/GSH ratio and GSH levels were also determined in human hepatic cell cultures.

As indicated in Table 3, nitric oxide-NO levels were determined spectrophotometrically or spectroflurometrically using commercial and non-commercial assays.

To our knowledge, no previous reviews on the involvement of oxidative stress in dengue pathogenesis have been performed. In the present systematic review, 32 articles concerning this subject were included.

Nitrite and ROS production by spleen cells of DENV-infected mice, and also after treatment with DENV-induced cytotoxic factor-CF or macrophage cytotoxin-CF2 have been observed both *in vivo* and *in vitro*.[22-24] These findings can be related to reports of significant increase of iNOS mRNA expression in spleen of DENV-infected wild-type mice.[25,26] In addition, temporal coincidence between iNOS upregulation and free radical production with hemorrhage development in DENV-infected mice have also been reported.[27]

These results are consistent with: (i) the significant reduction of hemorrhage development in wild-type and iNOS$^{-/-}$ mice after treatment with a NADPH oxidase inhibito,[27] (ii) the levels of oxidative stress biomarkers detected in DENV-infected mice in comparison to uninfected mice, [28] (iii) the association between viral presence and increased iNOS expression reported in another murine model,[29] and (iv) marked increase in susceptibility of iNOS-deficient mice to DENV infection.[25,26]

It is recognized that DENV-induced suppressor cytokine-SF binds to macrophages to transmit the suppressor signal to recruit the second subpopulation of suppressor T cells.[30] Khare et al.[31] demonstrated that NO and Ca^{2+} transmit the DENV-specific intracellular suppressor signal in macrophages.

The effects of DENV-derived oxidative stress and redox imbalance on human and animal cell cultures have been explored. NO, ROS, and reactive nitrogen species-RNS levels, GSSG/GSH ratio, iNOS gene expression and phosphorylation of STAT-1 were increased during *in vitro* infection. [27,28,32-39] However, NO production and the activation of these two transcription factors were blocked during antibody-dependent enhancement (ADE)-mediated DENV infection.[35]

Interestingly, inhibition of NO production was associated with secondary DENV infection in two *Macacus irus* inoculated with sequential dengue infection (DENV-4/DENV-2).[40]

In contrast, after exposition to DENV-3, no statically significant difference was found in the *ex vivo* NO production of peripheral blood cells obtained from dengue fever and dengue hemorrhagic fever patients. The authors explain this result by heterotypic dengue antibodies-mediated inhibitory effect on NOS.[41] This is consistent with disrupting the transcription of the iNOS gene transcription factor-IRF1 reported during ADE infection in human monocytic cells.[35]

RANTES mRNA expression in human hepatocyte-like cell line was abolished by antioxidants treatment[37] Additionally, antioxidants inhibited DENV-induced ROS production without affecting DENV-2 entry into human hepatocellular liver carcinoma cells.[28] However, N-acetyl-L-cysteine/NAC could reduce autophagy during DENV infection in human hepatoma cells.[42]

Moreover, IFN-g-induced NO production has been reported in DENV-infected human dendritic cells,[25] which is consistent with the absence of NO secretion by unstimulated DENV-infected monocytes/macrophages.[43]

It is important to note that DENV infection did not affect NO production in monocytes,[44] but the comparison of oxidant and antioxidant responses of monocytes from neonates, young adults and elderly subjects during an *in vitro* DENV infection showed that the induction of NO, lipid peroxidation, and CAT

activity, and GSH content were significantly lower in monocytes from neonates than in monocytes from adults and elderly.[45]

In regard to human platelets, their interaction with active or inactive virus did not have any effect on NO production.[46]

In comparison to uninfected cultures, the effects of different concentrations of a exogenous NO donor on DENV-infected culture were: (i) delayed expression of prM and NS1 genes, (ii) suppression of DENV replication,[47] and (iii) reduced levels of intracellular replicative species of DENV RNA. [48]

In addition, it has been shown that the replication of NO-sensitive DENV strains in human monocytic cells was significantly lower in comparison to NO-resistant strains. This difference disappeared after treatment with a selective inhibitor of iNOS, L-N6-(1-iminoethyl)-lysine/L-NIL.[49] This is consistent with the significant increase of DENV-2 RNA genome production in human monocytic cells after treatment with L-NIL.[35]

In the same way, the treatment with a competitive inhibitor of all three isoforms of NOS significantly increases DENV-antigen$^+$ monocytes frequency in comparison to untreated DENV-infected monocytes.[34]

On the other hand, the treatment with ROS and/or RNS inhibitors reversed the effect of DENV-2 infection or anti-NS1 IgG on human endothelial cells. [27-50] Similarly, pretreatment of this type of human cells with lipid raft-specific inhibitor or inhibitor of acid sphingomyelinase-aSMase inhibited anti-DENV NS1 antibody-induced NO production.[51]

After treatment of human endothelial cells with IgG fractions from dengue hemorrhagic fever patients containing high anti-NS1 antibody titers or with anti-NS1 monoclonal antibodies, intracellular ROS levels and heme oxygenase-1/HO-1 gene expression were significantly increased than in controls. [52]

Yen YT and Wu-Hsieh BA reported that apoptosis in endothelial cells transfected with NS2B-NS3 was higher in comparison with other viral components or more than endothelial cells transfected with vector alone.[53]

Recently, Olagnier et al.[54] have reported that nuclear factor-erythroid 2-related factor 2/Nrf-2 mediated oxidative stress response, iNOS signaling and production of NOS and ROS pathways were stimulated by DENV-2 infection of human monocyte-derived dendritic cells/Mo-DC. Also, a statistically significance decrease in SOD-2 mRNA levels was observed during treatment with ROS scavenger diphenyleneiodonium-DPI. In addition, these authors reported that DENV-2 infection was associated with NADPH oxidase-generated ROS accumulation.

In conclusion, a large body of *in vivo* and *in vitro* evidences showed that oxidative/nitrosative stress can be related to production of pathogenesis-related protein, increased susceptibility of mice to DENV infection, hemorrhage development in mice, cytokines and transcriptional factor expression, DENV replication, and apoptotic outcome in various human and animal cell cultures.

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