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'Nitric Oxide' A Dual Performer in Dengue Virus Infection

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ABSTRACT

Background and Aim: The study was conducted to assess the role of Nitric Oxide (NO) in the pathogenesis of Dengue fever (DF).

Materials and Methods: A total of 150 patients with positive Dengue serology and 50 candidates as controls in the age group of 15-65 years were included in the study. NO levels were measured by Griess Nitrate Method (Cadmium granules modified).

Results: The rise in NO was observed in 24 (20.86%) patients out of 115 DF patients, while it was raised in only 01 (2.8%) case out of 35 patients of DHF and the difference was observed to be statistically significant. NO level was within normal limits (Normal Range- 24.8-77.6µmol/L) in 92% (n=50) healthy controls and was raised in (NO value>77.66µmol/L) 4% healthy controls. A positive correlation was observed in the serum NO levels with platelet count. The majority of the patients in the study (41.33%) were in the age group of 21-30 years of age, followed by were < 20 yrs (30%) and 31-40 yrs (12.67%). Male:female ratio observed was 1.78:1. DF was diagnosed in 115 (76.67%) patients, while DHF was diagnosed in 35 (23.33%) patients, while no case of DSS was diagnosed.

Conclusion: NO plays a protective role in DF, while in DHF patients, the absence of rising NO levels may be one of the contributory factors leading to the progression of the disease and increased morbidity. NO levels can guide the physician about the evolution of a case of Dengue.

Keywords: Dengue Fever, Nitric Oxide, Dengue Hemorrhagic Fever, Griess Nitrite method

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1. Introduction

Dengue virus (DENV) is the cause of dengue fever and important mosquito-borne viral diseases. It has a significant impact on public health across the world (1). World Health Organization (WHO) data suggest that DENV transmission is endemic in more than 100 countries. It has been estimated that around 3.5 billion people, equivalent to 55% of the world's population, especially those in tropical and subtropical regions, are at risk. In a year, about 50 million suffer from DENV infections, out of which 500,000 people require hospitalization (2). The average mortality rate is around 5%, most of which are children and young adults (3, 4). The discovery of the ability of mammalian cells to synthesize the free radical Nitric Oxide (NO) has tremendously impacted further scientific research in various aspects of biology and medicine. NO is used as an important paracrine and autocrine signal by different types of cells, and it is produced by a variety of cells in the body, namely macrophages, vascular endothelial cells, Kupffer cells, adrenals, and cerebellar tissues (5). NO plays important functions in vivo, namely dilation of blood vessels, aggregation of platelets, and fighting against infections and tumors. It also acts as a mediator of inflammation and macrophage cytotoxic activity to transmit signals between nerve cells. Though NO has protective and

regulatory action in optimal concentration; however, higher concentrations have toxic effects **(6-8)**.

The role of NO in different viral infections has been an area of interest for many researchers for many years, and many interesting reviews have been published **(9, 10)**. Increased serum NO levels in DF but basal levels in DHF have been reported. Clinical and experimental data reveal that NO could be beneficial during dengue infection because of its antiviral and apoptotic effects; however, more intense investigations are needed **(11)**. Thus, the present study was conducted to study the role of NO in the Pathogenesis of DF.

2. Materials and Methods

The present study was a hospital-based cross-sectional study conducted from July 2016 to September 2018 in a tertiary care center in Pune, India. The study was approved by Institutional Ethics Committee (Research Protocol No. IESC/PG/020/16) dated 10.11.2016.

Study design

Study design: - The present study was a hospital-based cross-sectional study.

The study period was Two years (July 2016 to September 2018).

The study population includes patients with a positive serological test for Dengue and healthy controls.

Thus, two groups were formed in the study

Study group: 150 cases of Dengue

Control group: 50 healthy controls

Following inclusion and exclusion criteria were used to select the study group subjects.

Inclusion criteria for study group:

All patients with a positive serological test for Dengue (Positive ELISA test; kit used: J. Mitra). The principle of NS1, IgM, and IgG ELISA was the "Direct Sandwich principle", "MAC-capture ELISA", and "GAC-capture ELISA," respectively **(12, 13).** The manufacturer's kit instructions were followed for interpretation of the result, which were as follows:

- If Dengue NS1/IgM/IgG units < 9, the tests were interpreted as "Negative".
- If Dengue NS1/IgM/IgG units were between 9-11, the test was considered as "Equivocal".
- If Dengue NS1/IgM/IgG units were > 11, then the test was interpreted as "Positive" **(14-16)**.

The patients were classified as DF, DHF, and DSS according to WHO classification **(17, 18)**.

All patients were within the age group of 15 – 65 yrs.

Exclusion criteria for study group:

a) Presence of other co-infections malaria, enteric fever, tuberculosis, HIV & HBsAg

b) Immuno-compromised patients

c) Pregnant females

Following inclusion and exclusion criteria were used to select the study group subjects.

Inclusion Criteria for Control Group:

Age & Sex matched healthy controls were considered for the Control Group.

Exclusion Criteria for Control Group:

Subjects having other co-morbidities like Hypertension, Diabetes Mellitus, HIV, and chronic kidney disease (CKD) were excluded from the studies.

All these patients were tested for Dengue serology by ELISA, and patients with positive serological tests were included in the study.

The presence of other co-infections, malaria, enteric fever, tuberculosis, HIV & HBsAg, Immuno-compromised patients, and pregnant females were excluded from the study.

Age & Sex matched healthy controls without co-morbidities were considered for the Control Group.

The Griess Nitrite Method measured serum NO levels (Cadmium granules modified) **(5)**.

Test principle

The bio-molecule NO is short-lived, converting it to two stable products- nitrite (NO_2) and nitrate (NO_3). Cortas & Wakid invented the method, which includes the reduction of NO_3 to NO_2 by copper-coated cadmium granules **(19)**. In this method, nitrate is first reduced to nitrite, then treated with sulfanilamide and N-1-naphthyl-ethylene di-amine. A red color compound is formed after the absorption spectrum, which is determined by spectrophotometry. The concentration of nitrite was determined by regression analysis. The NO was estimated in terms of total nitrites in all the study patients and controls included in the present study.

Normal levels of NO- 24.8-77.6µmol/L **(20)**.

Data analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 16.0 software. Qualitative data: Frequencies were enlisted Quantitative data: - For continuous variables, Mean, Standard deviation.

3. Results

The majority of the patients in the study (41.33%) were in the age group of 21-30years of age, followed by <20yrs (30%) and 31-40yrs (12.67%). (Table 1). It was observed that 64% of patients were male and 36% were female, with a male: female ratio of 1.78:1.

DF was diagnosed in 115(76.67%) patients, DHF was diagnosed in 35 (23.33%) patients, while no case of DSS was diagnosed (Table 1-4).

Table 1. Age-wise distribution

Age group (Age in years)	No. of patients(n=150)	%
≤20	45	30.00
21-30	62	41.33
31-40	19	12.67
41-50	16	10.67
51-60	7	4.67
>60	1	0.67
Total	**150**	**100**

Table 2. Dengueserology result

Test (n=150)	Positive
	No. of patients (%)
NS1	94(62.67)
IgM	95(63.33)
IgG	84(56.00)

Table 3. Dengue serology dual marker positivity result

	NS1	IgG	IgM
NS1	44	13	03
IgG	3	08	39
IgM	13	39	09
Total	**60**	**60**	**51**

Table 4. Platelet count and severity of thrombocytopenia

Platelet count	No. of patients(n=150) (%)
<20000	3(2.00)
20000-50000	34(22.67)
50000-100000	36(24.00)
100000-150000	51(34.00)
150,000 - 450,000	26(17.33)
>450,000	00(0.00)
Total	150(100)

Dengue NS1 was positive in 62.67% of patients, IgM was positive in 63.33% of patients, and IgG was positive in 56% of patients. NS1+IgG were positive in 3 patients. NS1+IgM were positive in 13 patients. IgG + IgM were positive in 39 patients. NS1+IgM+IgG were positive in 34 patients.

It was seen that only NS1 was positive in 44 patients while only IgG was positive in 9 patients and only IgM

was positive in 8 patients. NS1+IgG were positive in 3 patients. NS1+IgM were positive in 13 patients. IgG + IgM were positive in 39 patients. NS1+IgM+IgG were positive in 34 patients. Thus, 65 (43.33%) patients were primary cases of Dengue, and 85 (56.66%) patients were secondary cases of Dengue.

Thrombocytopenia was seen in 82.67% of patients. Count between 50000-100000 was observed in 24% of patients, while a count between 20000-50000 was seen in 22.67% of patients. Platelet count was within normal limits in 17.33% of patients. Critically low platelet count was observed in only 2% of patients (Table 5).

Table 5. Nitric Oxide (NO) test results in study and control group

NO level (µmol/ L)	Study group(n=150)		Control group(n=50)	
	No. of patients	%	No. of patients	%
<24.8	0	0.00	00	0.00
24.8-77.6	125	83.33	46	92
>77.6	25	16.67	4	8

X^2 (Yates correction) = 1.627, df=1, p= 0.20211849 (statistically not significant)

It was observed that the NO level was increased in 8% cases in the control group (n=50) while it was raised in 16.67% (n=150) in the dengue patient group. The difference observed was statistically significant.

NO level was raised in 25 patients, while it was normal in 125 patients (Table 6).

Table 6. Association between dengue type and NO level

Dengue Type	NO Normal(%)(n=125)	NO Raised (%)(n=25)	Total (%)(n=150)
DF	91(72.8%)	24(96%)	115(76.6%)
DHF	34(27.2)	1(4%)	35(23.3%)
Total	**125**	**25**	**150**

X^2 (Yates correction) = 5.039, df=1, p= 0.02478279 (statistically significant)

The rise in NO was observed in 24(20.86%) patients out of 115 DF patients, while it was raised in only 01(2.8%) cases out of 35 patients of DHF and the difference was statistically significant.

4. Discussion

The present study was conducted in the "Department of Central Clinical Laboratory (CCL), of Dr. D. Y. Patil Medical College and Hospital and Research Centre, Pimpri, Pune." The study was conducted to assess the role of NO in the Pathogenesis of DF. A total of 150 patients with a positive serological test for Dengue were enrolled in the present study.

In the present study, it was observed that 64% of patients were male and 36% were female patients with a male: female ratio of 1.78:1. As men are more exposed to infected mosquitoes because of traveling time & working hours during daytime, there is slight preponderance compared to females in case of DENV infection. Our findings match with studies of Ashwini Kumar et al. **(20)** and Tejaswi et al. **(21)**.

The majority of the patients in the present study (41.44%) were in the age group of 21-30 years of age, followed by <20yrs (30%) and 31-40yrs (12.67%). Our findings agree with Thai et al., who observed that the "median age for DENV infection in primary and secondary infection is 9-20 years and 14-31 years respectively" **(19)**. Thus, it is observed that young males are affected more. This is because young males are exposed to different environments and are more likely to have mosquito bites than females and other age groups.

Thrombocytopenia was seen in 125(82.67%) cases in our study. (Table 4). Platelet count between 50000-100000/µL was observed in 24% of patients, while count between 20000-50000/µL was seen in 22.67% of patients. Platelet count was within normal limits in 25(17.33%) patients. Critically low platelet count (<20,000/µL) was observed in only 2% of patients, out of which 1(33%) patient suffered from DHF and the minimum platelet count noted in our study was 16,000/µL. Thrombocytopenia is caused by a virus's destruction of peripheral platelets or bone marrow megakaryocytes, which consequently reduces platelet production. The high DENV genome copies in platelets were directly correlated with the elevated platelet activation and the increased binding of complement factor C3 and IgG on their surface on day 4 **(22)**.

DF was diagnosed in 76.67% of patients, while DHF was diagnosed in 23.33% of patients, while no case of

DSS was diagnosed. Our results are closer to the study by Ashwini Kumar et al. **(20)**, who observed 83.9% with DF, 8.8% with DHF, and 7.3% with DSS. In a study conducted by Anjali S Raj et al. **(23)** study, DF patients were 49%, DHF patients were 40.8%, and DSS patients were10.2%. No case of DSS was diagnosed in our study. As the present study was conducted in the tertiary care institute, and the good patient care and adequate fluid replacement provided in the institute may be the possible reason for less detection rate of DSS.

Raised NO levels in 8% of cases of the control group may be because of some sub-clinical infections in apparently healthy subjects (Table 6). The difference observed was not statistically significant. It was seen that the rise in NO was observed in 24 (20.8%) patients with DF, while it was raised in only 1(2.8%) case of DHF, and the difference was observed as statistically significant (p=0.0247).

Rodhain and Rosen (1997) have discussed the clinical features of Dengue and the relation of NO with the severity of the disease **(24)**. Activation of DV-infected monocytes after stimulation of iNOS occurred both *in vivo* and *in vitro*, and the susceptibility of DV to NO production was also noted. NO inhibits aggregation, recruitment, and adhesion of platelets to the vascular endothelium.

Our study findings agreed with Valero et al. **(25)**. NO and reactive oxygen species elicit modulating effects on inflammation and regulating immune responses. During inflammatory reactions, monocytes & macrophages produce a greater number of oxidants, including NO. The increased production of NO in patients with DF is an expected finding because DENV is capable of inducing NO in cultures of splenic cells and Kupffer cells **(11).** Platelets can generate NO by stimulating NO synthetase **(26, 27)**.

5. Conclusion

Thus, regarding findings in the present study, it can be concluded that the rise in NO levels was statistically significant in DF patients as compared to DHF patients. NO plays a protective role in DF, while in DHF patients, the absence of rising NO levels may be one of the contributory factors leading to the progression of the disease and increased morbidity. NO levels can guide the physician about the evolution of a case of Dengue. The sample size of this study is small. Hence, a larger study and more investigations need to confirm the findings. It will also be interesting to study whether DHF patients will benefit from stimulating NO production in the future.

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Conflict of Interest

There are no conflicts of interest.

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