

Research Article

Oxidative and Chlorinative Stress in Children with Dengue Hemorrhagic Fever

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Available Online: 10th August, 2016

ABSTRACT

In this present study, we try to investigate the oxidative and chlorinative stress in children with Dengue Hemorrhagic Fever (DHF). The level of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA), myeloperoxidase (MPO) activity, and chlorinative index (CI), were measured in 61 confirmed dengue hemorrhagic (DHF) patients. Subjects were classified into 3 grades of DHF according to the World Health Organization (WHO) 1997 guidelines: grade I (DHF-1, n=22); grade II (DHF-2, n= 36); and grade III (DHF-3, n= 3). H₂O₂ and MDA level, and MPO activity were measured spectrophotometrically. CI was calculated by dividing the level of H₂O₂ and MPO activity. The results shows that the levels of H₂O₂ and MDA, MPO activity, and CI significantly different between group. The all parameters that investigated in this present study seems more higher with the higher grade of DHF, except for MPO activity. From this result, it can be concluded that both oxidative and chlorinative stress pathways might be involved in the pathomechanism of DHF.

Keywords: Dengue Hemorrhagic Fever, Oxidative Stress, Chlorinative Stress

INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is a major public health issue in developing countries, especially Indonesia¹. Indonesia is one of the nation in Southeast Asia that had the highest incidence of DHF. Since first discovered in 1968 in Surabaya, DHF cases in Indonesia have increased time by time². The national incidence rate has been 27 cases per 100,000 populations with a case fatality rate of 1.5%¹. Nowadays, all provinces in Indonesia have reported dengue cases, including South Kalimantan^{2,3}. The occurrence number of DHF in South Kalimantan was 30, 4%, while the mortality rate was 5 -8 %³. The DHF is caused by the infection of dengue virus (serotypes 1-4) that transmitted by the *Aedes aegypti* mosquito^{4,5}. Dengue virus is an RNA virus which belongs to the family Flaviviridae^{6,7}. The clinical spectrum of dengue fever ranges from a non-specific afebrile illness, a mild-form DHF, and dengue shock syndrome (DSS)^{7,8}. The pathomechanism of DHF is not clearly understood. Several hypotheses for the pathomechanisms of DHF have been proposed. Among them, oxidative stress might play a role in the pathomechanism of DHF^{9,10}. Several studies have reported that dengue virus mediated oxidative stress^{10,12}. It is well known that viral infection could activate neutrophils and others cells as a mechanism for protection^{11,13}. This activated neutrophils induced a process called respiratory burst. During this process, several reactive oxygen species (ROS) were produced. It

is caused by the increasing activity of NADPH oxidase and the releasing of myeloperoxidase (MPO)^{14,15}. NADPH oxidase was involved in the formation of several ROS such as, hydrogen peroxide (H₂O₂), while MPO was involved in the formation of another ROS such as, HOCl^{16,17}. The formation of HOCl will lead to a condition, known as chlorinative stress. It can be indicated by the increasing of ratio between H₂O₂ level and MPO activity known as Chlorinative Index (CI)¹⁸. Both oxidative and chlorinative stress will lead to a process called lipid peroxidation¹⁸. This process was characterized by the formation of some compounds, such as malondialdehyde (MDA)¹⁹. MDA is an indicator for ROS-induced lipid peroxidation^{20,21}. Since oxidative and chlorinative stress might be involved in the pathomechanism of DHF. Thus, our present study aimed to investigate the involvement of oxidative and chlorinative stress by measuring H₂O₂ and MDA level, MPO activity, and calculate the CI in the serum of DHF patient.

MATERIAL AND METHODS

Subjects

The study was conducted on DHF patients after informed consent was obtained from them. It was approved by the Ethics Commission of the Faculty of Medicine, Lambung Mangkurat University, Banjarbaru, South Kalimantan, Indonesia. A total of 61 subjects (aged 6 months–18 years) were recruited for this study. Subjects were divided into 3

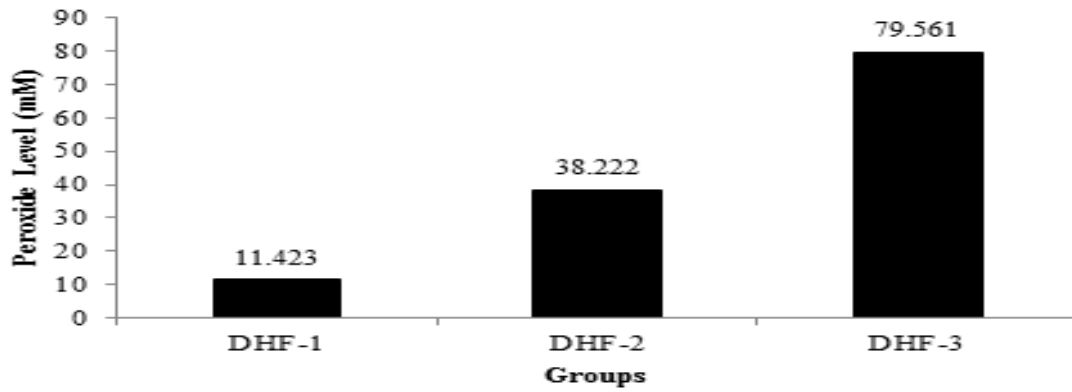


Figure 1: H₂O₂ level in the different stage of DHF (DHF: dengue hemorrhagic fever)

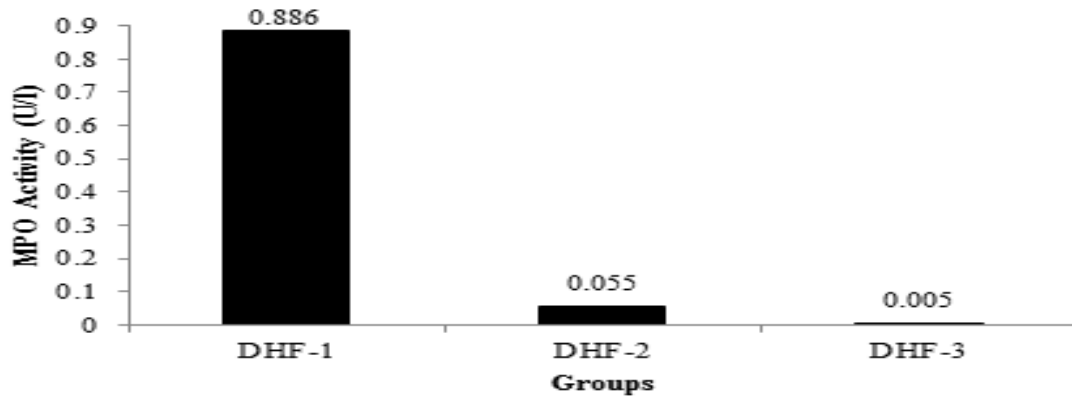


Figure 2: MPO activity in the different stage of DHF (MPO: myeloperoxidase; DHF: dengue hemorrhagic fever)

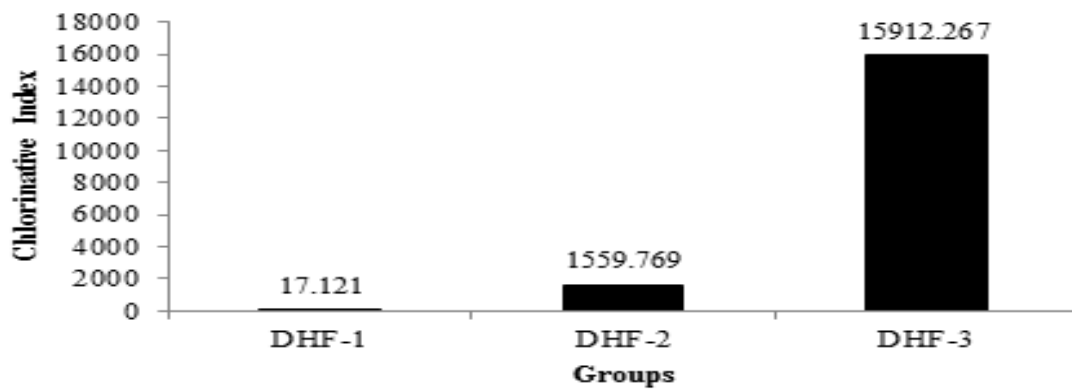


Figure 3: CI in the different stage of DHF (CI: chlorinative index; DHF: dengue hemorrhagic fever)

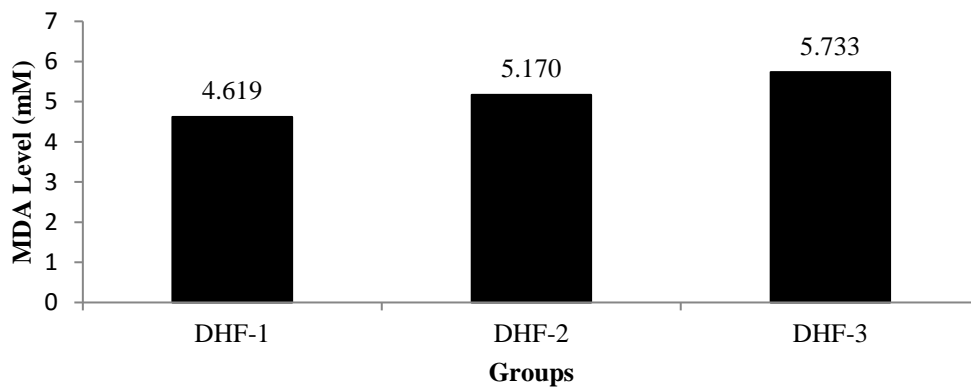


Figure 4: MDA level in the different stage of DHF (MDA: malondialdehyde; DHF: dengue hemorrhagic fever)

groups: group I (DHF-1, n = 22) with a grade I of DHF, while group II (DHF-2, n = 36) with grade II of DHF, and group III (DHF-3, n = 3) with grade III of DHF according to the World Health Organization (WHO) 1997 guidelines for control and prevention of DHF¹¹. Dengue infection was confirmed by IgM/IG g and NS-1 detection.

Samples collection

Samples of blood were collected from the patients using vacutainers containing EDTA. The samples were centrifuged for 15 min at 2000 rpm and stored at -2°C until analysis.

H₂O₂ level analysis

H₂O₂ level was calculated by the FOX2 method with slight modification. Solutions measured spectrophotometrically at $\lambda = 505$ nm. Standard and test solutions consisted of 1 M H₂O₂ 200 μ L and 200 μ L serum, respectively, with the addition of 160 μ L phosphate buffer solution pH 7.4, 160 μ L FeCl₃ (251.5 mg FeCl₃ dissolved in 250 ml distilled water) and 160 μ L o-fenantroline (120 mg o-phenantroline dissolved in 100 ml distilled water) for both solutions. The composition of the blank solution was identical to that of the test solution, except for the absence of FeCl₃ in the blank. Subsequent to preparation, all solutions were incubated for 30 minutes at room temperature, then centrifuged at 12,000 rpm for 10 minutes, and the absorbance of the standard (As), test (Au) and blank (Ab) solutions measured at $\lambda=505$ nm, using the supernatant of each solution²¹.

MPO activity analysis

MPO activity was measured spectrophotometrically using o-dianisidine (Sigma-Aldrich) and H₂O₂. In the presence of H₂O₂ as oxidizing agents, MPO catalyses the oxidation of o-dianisidine yielding a brown coloured product, oxidized o-dianisidine, with a maximum absorbance at 470 nm. One unit (U) of MPO activity was defined as that degrading 1 μ mol of H₂O₂ per minute at 25°C²².

CI analysis

CI was a ratio between H₂O₂ level and MPO activity. CI was calculated following to equation:

$$CI = \frac{\text{Hydrogen Peroxide Level}}{\text{MPO Activity}}$$

MDA level analysis

MDA level was calculated by thiobarbituric acid reactive substances (TBARS) by the technique already proposed by Buege and Aust²³. The supernatant were put into a Pyrex tube that contained 10% of trichloroacetic acid and 0.67% of TBARS and incubated at 100°C for 15 min. Then chill the mixture on ice for 5 min and add the 1.5 ml of n-butyl-liquor. Let the mixture stand for 40 s and centrifuged at 1000 rpm for 15 min. The TBARS value was calculated by the spectrophotometer at the absorbance of 532 nm and figured utilizing the coefficient 1.56×10^5 mol/cm. The MDA concentration expressed in μ mol MDA. As a standard solution we used commercially MDA.

Statistical analysis

Data are represented as mean \pm SEM. For comparing H₂O₂ and MDA levels, MPO activity, and CI between groups, Kruskal-Wallis followed by Mann-Whitney test were used. Statistical significance was set at $p < 0.05$. The

software used for the analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 16.0 and Microsoft Excel 2010 for Windows 10.

RESULTS

To observe the involvement of oxidative stress pathway in the dengue infection, the level of H₂O₂ was measured as an indicator of the oxidative stress status. The result shows that in all grades of DHF, H₂O₂ was produced (Figure 1). Kruskal-Wallis test result shows that the level of H₂O₂ is significantly different from each other ($p < 0.05$). Mann-Whitney test results show that there are a significant difference of H₂O₂ level between DHF-1 and DHF-2 group, DHF-1 and DHF-3 group, and DHF-2 and DHF-3 group ($p < 0.05$) (Table 1). The result indicated that oxidative stress might be involved in DHF and the response is higher in severe cases.

^ap-Values were calculated using the Kruskal–Wallis test followed by Mann-Whitney test; $p < 0.05$ was considered statistically significant.

^b Indicates p-value when compared between DHF-1 and DHF-2.

^c Indicates p-value when compared between DHF-1 and DHF-3.

^d Indicates p-value when compared between DHF-2 and DHF-3.

Plasma MPO activity was found to be lower in the case with the severe forms (DHF-2 and DHF-3) than in DHF-1 cases (Figure 2). Analysis of the mean comparison between MPO activity in different grade of DHF revealed a significant difference between all grades of DHF ($p < 0.05$). Mann-Whitney test results shows that there are a significant difference of MPO activity between DHF-1 and DHF-2 group, DHF-1 and DHF-3 group, and DHF-2 and DHF-3 group ($p < 0.05$) (Table 1). The result indicated that chlorinative stress might be involved in DHF. Fig. 3 represented the mean values \pm standard error (mean \pm SE) of CI. Dispersion of measured values around each mean varied from 17.121 to 15912.267. That data also suggests that the highest CI is in the DHF-3 group and the lowest is the DHF-1 group (Figure 3). Statistical analysis test results show that all groups of treatment significantly ($p < 0.05$) different from each other. Mann-Whitney test results show that there are a significant difference of CI between DHF-1 and DHF-2 group, DHF-1 and DHF-3 group, and DHF-2 and DHF-3 group (table 1). The result is in line with the result in figure 2. It is indicated that chlorinative stress might be involved in DHF. Fig. 4 represented the mean values \pm standard error (mean \pm SE) of plasma MDA levels. Dispersion of measured values around each mean varied from 4.619 to 5.733. That data also suggests that the highest MDA level is in the DHF-3 group and the lowest is the DHF-1 group (Figure 4). Statistical analysis test results show that all groups of treatment significantly ($p < 0.05$) different from each other. Mann-Whitney test results show that there are a significant difference of MDA level between DHF-1 and DHF-2 group, DHF-1 and DHF-3 group, and DHF-2 and DHF-3 group ($p < 0.05$) (Table 1). The result indicated that DHF increased lipid peroxidation.

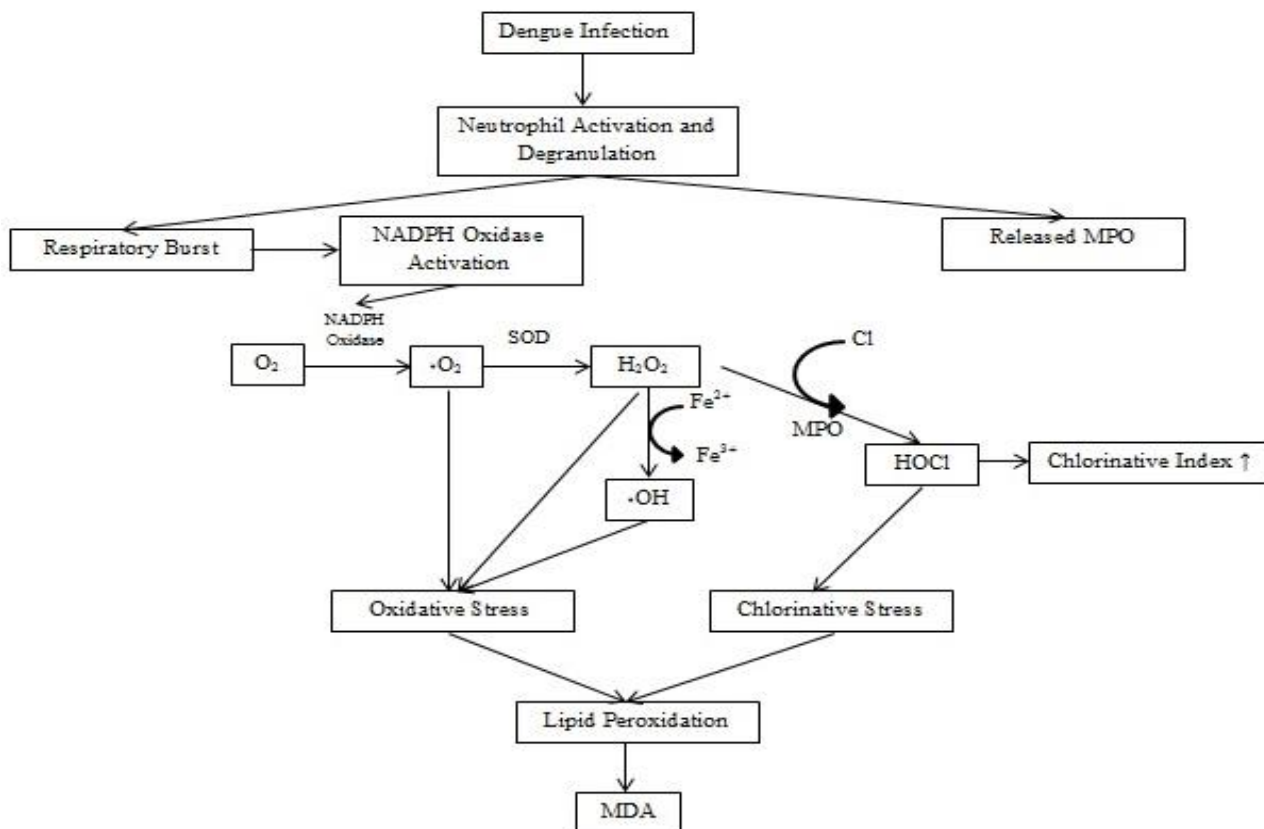


Figure 5: Oxidative and chlorinative stress in DHF pathomechanism.

DISCUSSION

The present study compared the plasma levels of H_2O_2 in the different grade of dengue infection (grade I, II, and III). The result shows that the H_2O_2 level was found to be higher in grade III than grade I and II. This result of this present study suggests that dengue virus infection induced the formation ROS. ROS seems to be higher in the grade III more than grade I, and II. This result indicated that the level of ROS will be increase with the increasing of DHF severity. The result is similar to those reported previously in dengue-infected Cuban adult, in whom serum level of total H_2O_2 was significantly higher in DHF patients than in normal healthy individuals¹¹. The increasing of ROS formation may be due to a process known as respiratory burst. Respiratory burst is a term used when neutrophils become activated through the actions of NADPH oxidase and generate ROS production that can be very effective to kill a microorganism, inducing virus²⁴. This mechanism has been described in several studies for several viral infections, such as hepatitis C virus, rhinovirus, HIV, and dengue virus²⁵. Besides ROS, activated neutrophils several components and chemokines. One of a major component that found in azurophilic granules of neutrophils is MPO. Our result study indicated that MPO is involved in the pathomechanism of DHF. The result shows that the MPO activity is lower in the higher grade of DHF. This is in contrast to previous reports, but there has been limited data available on the effect of DHF on MPO activity²⁶. This different result could be due to the severity of the disease. It is well known that DHF is characterized by

trombocytopenia and leucopenia. It has also been reported that dengue infections can caused neutropenia. The severity of neutropenia is directly proportional to the severity of DHF. Khan *et al* result study shows that neutropenia was seen more commonly in patients with DHF than patients with DF²⁷. Since MPO activity reflects the neutrophil functions, lower neutrophil count might be affected the MPO activity²⁸. The MPO is a glycosylated haem enzyme that participates in innate immune defense by forming microbicidal reactive oxidants and diffusible radical species^{17,29}. MPO catalyzes the reaction between H_2O_2 and chloride ions to form HOCl, a highly oxidising agent, to kill intracellular microorganisms²⁹. HOCl can cause a condition known as chlorinative stress³⁰. This condition can be seen by an index known as CI. Our result suggests that CI is increasing with the increasing grade of DHF. This result indicated that in the higher degree of DHF, the chlorinative stress that occurred will be higher than the lower grade. Dengue viral infections will lead to an activation and degranulation of neutrophils. This activation and degranulation induced the activity of NADPH oxidase to catalyst O_2 to form $\cdot\text{O}_2^-$ via respiratory burst. $\cdot\text{O}_2^-$ will dismutated by SOD to form H_2O_2 and with the presence of some transition metals, H_2O_2 were breakdown to $\cdot\text{OH}$ via fenton reaction. All ROS that produced during respiratory burst can cause oxidative stress. Besides activated NADPH oxidase, the activation and degranulation of neutrophil can induce the releasing of MPO. Then, MPO catalyst the reaction between H_2O_2 and Cl to form HOCl. This will lead to a condition known as

Table 1: Comparison of H₂O₂ and MDA levels, MPO activity, and CI in the different stage of DHF^a

Parameters	DHF-1	DHF-2	DHF-3	p-Value ^b	p-Value ^c	p-Value ^d
Peroxide (mM)	11.423	38.222	79.561	0.000	0.006	0.005
MDA (mM)	4.619	5.170	5.733	0.000	0.006	0.007
MPO (U/l)	0.886	0.055	0.005	0.000	0.006	0.004
CI	17.121	1559.77	15912.267	0.000	0.006	0.004

H₂O₂: hydrogen peroxide; MDA: malondialdehyde; MPO: myeloperoxidase; CI: chlorinative index; DHF: dengue hemorrhagic fever.

chlorinative stress which is characterized by an increase of Chlorinative Index (CI). Both oxidative and chlorinative stress can induce a lipid peroxidation which is marked by the formation of MDA. Oxidative and chlorinative stress, both has a consequence. The consequence is macromolecul damage, such as lipid. Lipid damage caused by both oxidative and chlorinative stress known as lipid peroxidation^{18,30}. One of the most well studied markers of lipid peroxidation is MDA^{19-20,31}. Consistent with this idea, our result data suggest that MDA was formed during dengue infection. In higher grade, the level of MDA seems to be more higher. It may be cause this MDA level are correlated with the level of H₂O₂ and CI rate, although this study does not correlated these variables. The whole process was illustrated in figure 5. In conclusion, the present study demonstrated that oxidative and chlorinative stress might be involved in pathomechanism of DHF. It seems the oxidative and chlorinative stress is more higher with the higher grade of DHF. From the results, it also can be concluded that both oxidative and chlorinative stress could cause a further reaction to damage macromolecul, such as lipid.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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