Oxidative and Chlorinative Stress in Children with Dengue Hemorrhagic Fever

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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= 36); grade II (DHF-2, 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 show that the levels of H2O2 and MDA, MPO activity, and CI significantly different between group. All the parameters that investigated in this present study seem 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 Indonesia. Indonesia is one of the nations 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 time. The national incidence rate has been 27 cases per 100,000 populations with a case fatality rate of 1.5%. Nowadays, all provinces in Indonesia have reported dengue cases, including South Kalimantan. The occurrence number of DHF in South Kalimantan was 30; 4%, while the mortality rate was 5-8%. The DHF is caused by the infection of dengue viruses (serotypes 1-4) that transmitted by the Aedes aegypti mosquito. Dengue virus is an RNA virus which belongs to the family Flaviviridae. The clinical spectrum of dengue fever ranges from a non-specific afebrile illness, a mild-form DHF, and dengue shock syndrome (DSS). 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. Several studies have reported that dengue virus mediated oxidative stress. It is well known that viral infection could activate neutrophils and other cells as a mechanism for protection. 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). 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. The formation of HOCl will lead to a condition, known as chlorinative stress. It can be indicated by the increasing ratio between H2O2 level and MPO activity as known Chlorinative Index (CI). Both oxidative and chlorinative stress will lead to a process called lipid peroxidation. This process was characterized by the formation of some compounds, such as malondialdehyde (MDA). MDA is an indicator for ROS-induced lipid peroxidation. 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, Banjarmasin, 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: H$_2$O$_2$ 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 DHF. 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 λ = 505 nm. Standard and test solutions consisted of 1 M H₂O₂ 200 µL and 200 µL serum, respectively, with the addition of 160 µL phosphate buffer solution pH 7.4, 160 µL FeCl₃ (251.5 mg FeCl₃ dissolved in 250 ml distilled water) and 160 µL 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 (At) and blank (Ab) solutions measured at λ = 505 nm, using the supernatant of each solution.

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 μmol of H₂O₂ per minute at 25°C.

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

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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 Aust. The supernatant were put into a Pyrex tube that contained 10% of trichloracetic 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 x 105 mol/cm. The MDA concentration expressed in μmol MDA. As a standard solution we used commercially MDA.

Statistical analysis
Data are represented as mean±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.

P-values were calculated using the Kruskal–Wallis test followed by Mann-Whitney test; p < 0.05 was considered statistically significant.

a) Indicates p-value when compared between DHF-1 and DHF-2.
b) Indicates p-value when compared between DHF-1 and DHF-3.
c) 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 show that there is 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 represents the mean values±standard error (mean±SE) of CI. Dispersion of measured values around each mean varied from 17.121 and 159.12.267. 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 (p<0.05) (Table 1). The result indicated that DHF increased lipid peroxidation.
DISCUSSION
The present study compared the plasma levels of \( \text{H}_2\text{O}_2 \) in the different grade of dengue infection (grade I, II, and III). The result shows that the \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) was significantly higher in DHF patients than in normal healthy individuals. 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 virus. This mechanism has been described in several studies for several viral infections, such as hepatitis C virus, rhinovirus, HIV, and dengue virus. 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 activity. 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 DF. Since MPO activity reflects the neutrophil functions, lower neutrophil count might be affected the MPO activity. The MPO is a glycosylated haem enzyme that participates in innate immune defense by forming microbicidal reactive oxidants and diffusible radical species. MPO catalyzes the reaction between \( \text{H}_2\text{O}_2 \) and chloride ions to form HOCl, a highly oxidising agent, to kill intracellular microorganisms. HOCl can cause a condition known as chlorinative stress. 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 \( \text{O}_2 \) to form \( \cdot\text{O}_2 \) via respiratory burst. \( \cdot\text{O}_2 \) will dismutated by SOD to form \( \text{H}_2\text{O}_2 \) and with the presence of some transition metals, \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) and CI to form HOCl. This will lead to a condition known as...
Table 1: Comparison of H$_2$O$_2$ and MDA levels, MPO activity, and CI in the different stage of DHF$^a$

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DHF-1</th>
<th>DHF-2</th>
<th>DHF-3</th>
<th>p-Value$^b$</th>
<th>p-Value$^c$</th>
<th>p-Value$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide (mM)</td>
<td>11.423</td>
<td>38.222</td>
<td>79.561</td>
<td>0.000</td>
<td>0.006</td>
<td>0.005</td>
</tr>
<tr>
<td>MDA (mM)</td>
<td>4.619</td>
<td>5.170</td>
<td>5.733</td>
<td>0.000</td>
<td>0.006</td>
<td>0.007</td>
</tr>
<tr>
<td>MPO (U/l)</td>
<td>0.886</td>
<td>0.055</td>
<td>0.005</td>
<td>0.000</td>
<td>0.006</td>
<td>0.004</td>
</tr>
<tr>
<td>CI</td>
<td>17.121</td>
<td>159.77</td>
<td>15912.267</td>
<td>0.000</td>
<td>0.006</td>
<td>0.004</td>
</tr>
</tbody>
</table>

H$_2$O$_2$: 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 lipoperoxidation which is marked by the formation of MDA. Oxidative and chlorinative stress, both has a consequence. The consequence is macromolecule damage, such as lipid. Lipid damage caused by both oxidative and chlorinative stress known as lipid peroxidation. One of the most well studied markers of lipid peroxidation is MDA. 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 H$_2$O$_2$ 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 macromolecule, such as lipid.

CONFLICT OF INTEREST
We declare that we have no conflict of interest.

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