Chlorinative Index in Liver Toxicity Induced by Iron

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ABSTRACT
Iron (Fe) is a metal that is present in high concentrations in coal dust. Exposure to coal dust could increase the concentration of Fe in the body and can cause several health problems, including liver cells damaged. However, the mechanism of liver cell damage by Fe in the coal dust is still not clear. In this present study, we proposed that Fe could induced liver cells damaged via chlorinative stress pathway. Thus, our study aimed to investigated the effect of Fe on liver cells via chlorinative stress pathway by measuring the levels of hydrogen peroxide (H₂O₂) and Advanced Oxidation Protein Products (AOPPs), the myeloperoxidase (MPO) activity, and chlorinative index (CI). The results revealed that Fe exposure could significantly increased H₂O₂ and AOPPs level, and CI in liver cells (P<0.05). The result also revealed that Fe exposure could decreased the MPO activity but non-statistically significant (P>0.05). These results indicated that Fe exposure induce chlorinative stress which can be seen from the increasing of H₂O₂ and AOPPs levels, and CI. However, the exact mechanism of Fe induced the chlorinative stress is still unclear yet, because the MPO activity did not statistically changed. Further study may be needed to determine the Fe mechanism to induce chlorinative stress.

Keywords: Chlorinative Stress, Coal Dust, Iron.

INTRODUCTION
South Kalimantan is known as one of the most coal-rich states of Indonesia. South Kalimantan produced roughly 79 Mt in 2008 and rising to 118 Mt in 2011 (33% of national output). According to Greenpeace’s spatial analysis, official mining concessions cover approximately 1 million hectares (Mha) of South Kalimantan’s total area of 3.7 Mha. However, besides economical benefits, it has provokes severe environmental problems¹. Coal mining generate air pollution, primarily particulate matter, through blasting, wind erosion of exposed areas, and handling of coal at the mines, during transportation, and at processing plants². Particulate matter is a general term to describe small particles in the air, of which coal dust is one type. Particulate matter is toxic to human beings because it can enter the bloodstream after being inhaled³. According to Setiawan et al⁴ study, the coal dust contained several metal, with the highest level is iron (Fe). This Fe can enter the human body via particulate matter and can cause several health complications including liver cells damage⁵. Sambandam et al⁶ study demonstrated that coal fly ash particulate matter induced cytotoxicity, oxidative stress, DNA damage, and apoptosis in human liver cells. Several reports indicated that Fe-induced liver damaged were related to oxidative stress⁷. However, in this present study we try to demonstrate the Fe-induced liver damaged via chlorinative or chlorinative stress pathway. The term of chlorinate or chlorinative stress is use to describe when some chlorinated oxidants such as hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypoiodous acids (HOSCn) were formed after oxidation by myeloperoxidase (MPO) with the presence of hydrogen peroxide (H₂O₂)⁸⁻¹¹. The chlorinated oxidants that produced during chlorinative stress condition are capable causing damage to macromolecules including protein. Protein damage as a result of chlorinative stress can be measured using Advanced Oxidation Protein Products (AOPPs) which is known as a marker of that condition¹². Furthermore, chlorinative stress can also be measured using chlorinative index (CI). Considering the chlorinative stress may be involve in Fe-induce liver cells damage, the present study was performed to evaluate of Fe on chlorinative stress induced liver cells damaged by measuring the H₂O₂ and AOPPs levels, MPO activity, and CI in vitro.

MATERIAL AND METHODS
Animals and Homogenate Preparation
Male rats (Rattus novergicus) weighing 200–250 gram with 2-3 months old were obtained from the Abadi Jaya farm at Yogyakarta, Indonesia, in healthy condition. The experiment was approved by the Ethical Committee from the Lambung Mangkurat University, South Kalimantan, Indonesia. Animals were fed under standard conditions and acclimatized with a 12 hours light/dark cycle. The

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animals were sacrificed by surgical procedure and the livers was removed. Then, the organs homogenized in phosphate buffer saline (pH 7.0) and was ready to used for in vitro experimental models.

Experimental Models
Homogenate samples were divided into 4 groups (1 control group and 3 treatment groups). Control (T0) group: liver homogenate only; Treatment 1 (T1) group: liver homogenate + 30 µg/dl of ferrous sulphate (FeSO4); Treatment 2 (T2) group: liver homogenate + 300 µg/dl of ferrous sulphate (FeSO4); Treatment 3 (T3) group: liver homogenate + 3000 µg/dl of ferrous sulphate (FeSO4). Each solution then incubated at 37°C for 1 hour. After incubation, liver H2O2, and AOPPs level, MPO activity, and CI were estimated.

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 PBS 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 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 at 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 agent, 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 the degrading 1 µmol of H2O2 per minute at 25°C.

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

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

AOPPs concentration analysis
AOPPs concentration analysis were calculated by spectrophotometric methods which was first performed by Witko-Sarsat et al.15, with slight modification. 200 µl of supernatant from the liver homogenate were diluted with phosphate buffer solution. Then, placed on 96-test wells. Add 20 ml of acetic acid in each test well. For the standard, add 10 ml of 1.16 mol potassium iodide, 200 ml of chloramine-T solution (0–100 mmol/l), and 20 ml of acetic acid. Placed the standard mixture into standard wells. Then, read the absorbance of the mixture at 340 nm. The absorbance was read against a blank solution. A blank solution is a mixture between 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. AOPPs concentrations were expressed as µmol/l of chloramine-T equivalents.

Statistical analysis
Statistical analysis was performed using SPSS for Windows version 16.0. Data were checked for normality (Shapiro–Wilk normality test) and homogeneity of variance (Levene’s test). Then, data was divided in to two assumptions. The normally and homogeneity distributed data were run with oneway analysis of variance (ANOVA) and followed by post hoc Tukey HSD test. The non-normally and non-homogeneity distributed data were run with the non-parametric Kruskal-Wallis Test and followed by Mann-Whitney U test. P-values <0.05 were considered statistically significant.

RESULTS
In this present study, the level of liver H2O2 with the presence of Fe in different concentrations was investigated. The result shows in figure 1. Fe exposure caused the distortion in the liver H2O2 levels. After Fe exposure, there was a significant difference in the H2O2 level between group of treatments (ANOVA test, p<0.05) (table 1). A liver concentrations of H2O2 was significantly higher in all group of treatments compare to control (p<0.05). Post Hoc Tukey HSD test results show that there are significant differences between between group T0 and T3, and T1 and T3 (table 1). MPO activity in the liver that received different doses of Fe and the controls are presented in the Figures 2. After the administration Fe, MPO activity seems to be decreased compared to the controls (Figure 2). There was a dose dependent decrease in MPO activity in the liver tissues. However, statistical analysis test results shows that the decreasing of MPO activity were not statistically significant (Kruskal-Wallis test, P>0.05). The effect of different dose of Fe on CI is presented in figure 3. It was observed that the CI increased in all Fe treatment groups as compared to control group. The increase in CI were in a dose dependent manner. ANOVA test results shows that there is a significance difference in CI between group of treatments (p<0.05) (table 1). Post Hoc Tukey HSD test shows that there are significant differences between between group T0 and T3, and T1 and T3 (table 1). The effect of different dose of Fe on AOPPs level is presented in figure 4. It was observed that the AOPPs level increased in all Fe treatment groups as compared to control group. The increase in AOPPs level were in a dose dependent manner. ANOVA test results shows that there is a significance difference in AOPPs between group of treatments (p<0.05) (table 1). Post Hoc Tukey HSD test shows that there are significant differences between between group T0 and T3, and T1 and T3 (table 1).

DISCUSSION
Fe is a critical component in many metabolic functions. Fe has a unique electrochemical property, which can make the Fe can act as an ideal redox active cofactor. The redox reactivity of Fe makes it extremely useful, but the
Table 1: Comparison of $\text{H}_2\text{O}_2$ and AOPPs levels, MPO activity, and CI in the different group of treatments.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>p-Value$^b$</th>
<th>P-Value$^c$</th>
<th>P-Value$^d$</th>
<th>P-Value$^e$</th>
<th>P-Value$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>8.551</td>
<td>8.605</td>
<td>8.802</td>
<td>9.736</td>
<td>0.983</td>
<td>0.382</td>
<td>0.002</td>
<td>0.567</td>
<td>0.003</td>
</tr>
<tr>
<td>MPO</td>
<td>0.086</td>
<td>0.030</td>
<td>0.011</td>
<td>0.006</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AOPPs</td>
<td>0.269</td>
<td>0.603</td>
<td>0.987</td>
<td>1.628</td>
<td>0.292</td>
<td>0.014</td>
<td>0.000</td>
<td>0.198</td>
<td>0.002</td>
</tr>
<tr>
<td>CI</td>
<td>111.026</td>
<td>331.489</td>
<td>822.435</td>
<td>1629.419</td>
<td>0.806</td>
<td>0.077</td>
<td>0.001</td>
<td>0.264</td>
<td>0.003</td>
</tr>
</tbody>
</table>

$\text{H}_2\text{O}_2$: Hydrogen Peroxide; MPO: Myeloperoxidase; AOPPs: Advanced Oxidation Protein Products CI: Chlorinative Index; T0: control group; T1: treatment with 30 µg/dl; T2: treatment with 300 µg/dl; T3: treatment with 3000 µg/dl.

$^a$p-Values were calculated using the One Way ANOVA or Kruskal–Wallis test and followed by Post Hoc Tukey HSD or Mann-Whitney test; p < 0.05 was considered statistically significant.

$^b$ Indicates p-value when compared between T0 and T1.

$^c$ Indicates p-value when compared between T0 and T2.

$^d$ Indicates p-value when compared between T0 and T3.

$^e$ Indicates p-value when compared between T1 and T2.

$^f$ Indicates p-value when compared between T2 and T3.

Figure 1: Comparison of $\text{H}_2\text{O}_2$ level between a group of treatments.

Figure 2: Comparison of MPO activity between a group of treatments.

Figure 3: Comparison of CI between a group of treatments.
same property makes it a toxic entity, because of its propensity to generate Reactive Oxygen Species (ROS) if it is not tightly bound and/or it is present in excess\(^6\). This in line with the result of this present study. The result showed that Fe exposure could significantly increase the level of \(\text{H}_2\text{O}_2\) in liver cells, but in the higher concentration (3000 \(\mu\)g/dl). It is well known that Fe in excessive amounts may be toxic particularly to the liver since it is the major site of Fe storage\(^7\). The basic mechanism by which excess Fe may be toxic include the formation of ROS. Fe could generate ROS formation through several mechanisms as follow:

- Fenton reaction. In the fenton reaction Fe\(^{2+}\) is oxidized to Fe\(^{3+}\) by oxygen to form radical superoxid. Then, radical superoxide reacts with hydrogen to form \(\text{H}_2\text{O}_2\). Furthermore, \(\text{H}_2\text{O}_2\) could reacts again with Fe\(^{2+}\) and radical hydroxyl is produced\(^7,18-20\)

- Haber-Weiss reaction. Superoxide and \(\text{H}_2\text{O}_2\) that produced during fenton reaction could react to form hydroxyl radical which promote another reaction to form another ROS, etc\(^2\).

\(\text{H}_2\text{O}_2\) which produced by Fe can be use by MPO to oxidized chlorinated oxidants\(^22\). From this point of view, we hypothesized that Fe exposure could affect MPO activity and increased the CI. Interestingly, the results of this present study indicated that Fe exposure was not significantly alter the MPO activity but significantly increased CI in the liver cells especially if the Fe concentration is excessive. However, to best of our knowledge, there have been no investigations of association between Fe exposure, MPO activity, and CI in liver cells. This is the first study to examine the effects of Fe exposure on MPO activity and CI in liver cells. The results also indicated, even the MPO activity do not alter in the higher concentration is excessive. This results suggest that Fe still induced chlorinative stress. This result is similar to Setiawan \textit{et al.}\(^23\) study. That results study shows that coal dust exposure which Fe is contained in the coal dust particle can cause chlorinative stress, but in a plasma of rats. However, further investigation is needed to elucidate the mechanism of action of Fe on increasing the CI. In this present study we also assumed that Fe promotes \(\text{H}_2\text{O}_2\) formation and affect MPO activity and CI which promotes a condition known as chlorinative stress in liver cells. This condition have several consequences such as, protein damages which can be seen from AOPPs level. AOPPs formed as a result of irreparable oxidative damage to the proteins, are defined as novel, reliable markers of irreversible oxidative damage\(^24\). The results indicated that AOPPs level increased significandy in liver cells especially when Fe level is excessive. This results also similar with Setiawan \textit{et al.}\(^23\) study which has been mentioned in the previous paragraph. The results also indicated that coal dust which Fe is contained in the coal dust particle could increased AOPPs level, but in plasma of rats.

**CONFLICT OF INTEREST**

We declare that we have no conflict of interest.

**REFERENCES**


![Figure 4: Comparison of AOPPs level between a group of treatments.](image_url)


