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Research Article

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 (H_2O_2) and Advanced Oxidation Protein Products (AOPPs), the myeloperoxidase (MPO) acivity, and chlorinative index (CI). The results revealed that Fe exposure could significantly increased H_2O_2 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 H_2O_2 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 problems[1]. 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 plants[2]. 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 inhaled[3]. According to Setiawan *et al*[4] 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 damage[5]. Sambandam *et al*[6] 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 stress[5,7,8]. However, in this present study we try to demonstrate the Fe-induced liver damaged via chlorinated 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 hypothiocyanous acids (HOSCN) were formed after oxidation by myeloperoxidase (MPO) with the presence of hydrogen peroxide (H_2O_2)[9-11]. 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 condition[12]. 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 H_2O_2 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 ($FeSO_4$); Treatment 2 (T2) group: liver homogenate + 300 µg/dl of ferrous sulphate ($FeSO_4$); Treatment 3 (T3) group: liver homogenate + 3000 µg/dl of ferrous sulphate ($FeSO_4$). Each solution then incubated at 37°C for 1 hour. After incubation, liver H_2O_2, and AOOPs level, MPO activity, and CI were estimated.

H_2O_2 level analysis

H_2O_2 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 H_2O_2 200 µL and 200 µL serum, respectively, with the addition of 160 µL PBS pH 7.4, 160 µL $FeCl_3$ (251.5 mg $FeCl_3$ 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 $FeCl_3$ 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 λ=505 nm, using the supernatant of each solution[13].

MPO activity analysis

MPO activity was measured spectrophotometrically using o-dianisidine (Sigma-Aldrich) and H_2O_2. In the presence of H^2O_2 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 that degrading 1 µmol of H_2O_2 per minute at 25°C[14].

Chlorinative index analysis

Chlorinative index was a ratio between H_2O_2 level and MPO activity. Chlorinative index was calculated following to equation:

$$CI = Hydrogen\ Peroxide\ Level / M\ P\ O\ Activity$$

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[15].

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 the parametric one-way analysis of variance (ANOVA) and followed by post hoc Tuckey HSD test. The non-normally and /or 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 H_2O_2 with the presence of Fe in different concentrations was investigated. The result shows in figure 1. Fe exposure caused the distortion in the liver H_2O_2 levels. After Fe exposure, there was a significant difference in the H_2O_2 level between group of treatments (ANOVA test, p<0,05) (table 1). A liver concentrations of H_2O_2 was significantly higher in all group of treatments compare to contol (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 H_2O_2[a] and AOPPs[a] levels, MPO activity, and CI[a] in the different group of treatments.

Parameters	T0	T1	T2	T3	p-Value[b]	P-Value[c]	P-Value[d]	P-Value[e]	P-Value[f]
H_2O_2	8,551	8,605	8,802	9,736	0.983	0.382	0.002	0.567	0.003
MPO	0,086	0,030	0,011	0,006	NS	NS	NS	NS	NS
AOPPs	0,269	0,603	0,987	1,628	0.292	0.014	0.000	0.198	0.002
CI	111,026	331,489	822,435	1629,419	0.806	0.077	0.001	0.264	0.003

H_2O_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.

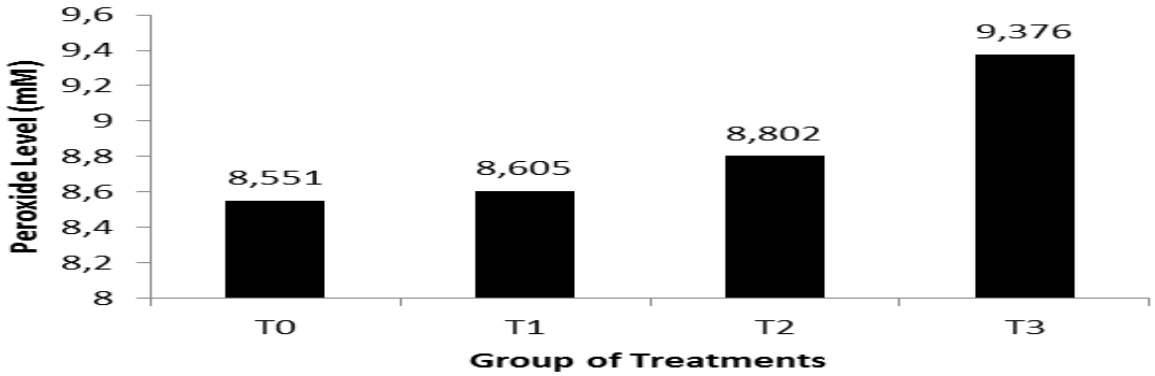

Figure 1: Comparison of H_2O_2 level between a group of treatments.

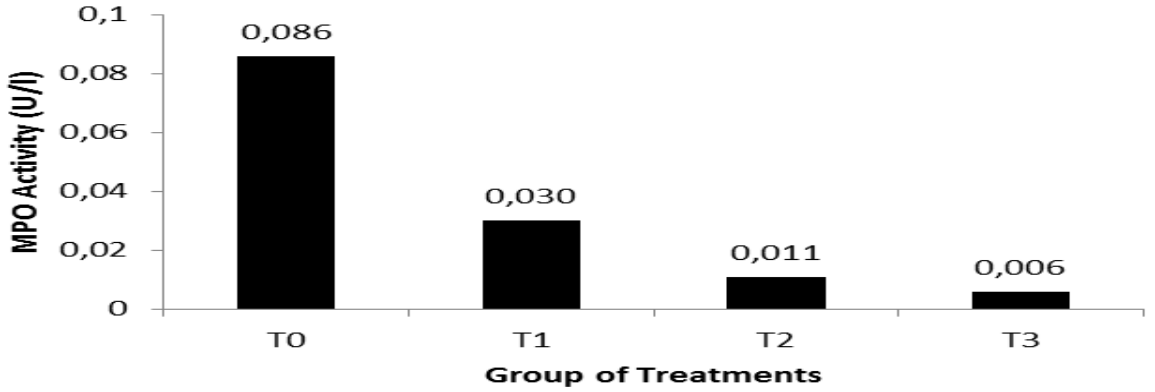

Figure 2: Comparison of MPO activity between a group of treatments.

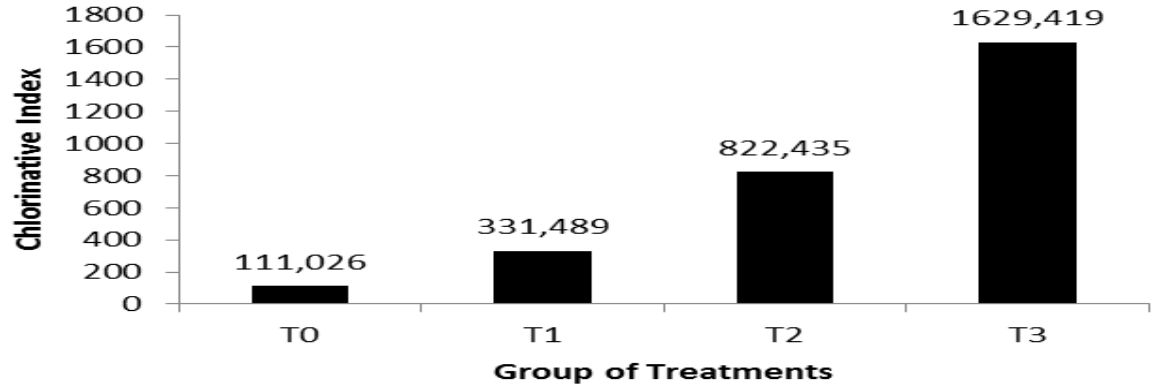

Figure 3: Comparison of CI between a group of treatments.

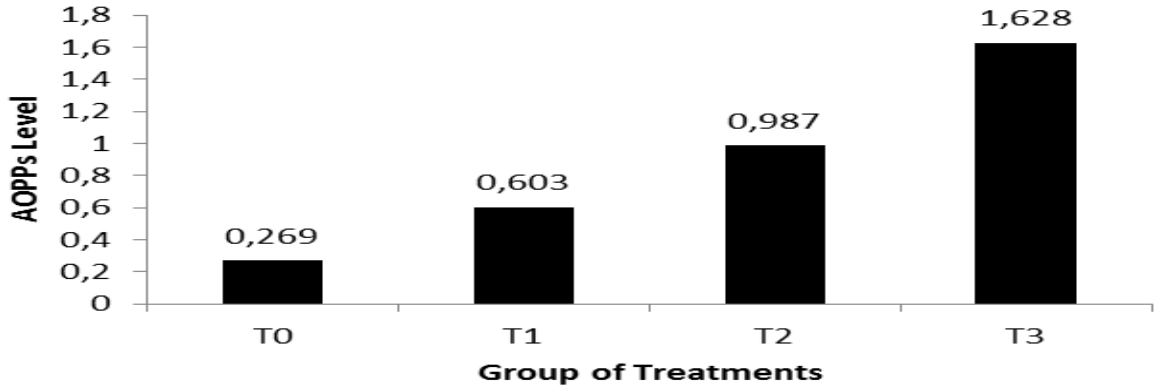

Figure 4: Comparison of AOPPs level between a group of treatments.

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[16]. This in line with the result of this present study. The result showed that Fe exposure could significantly increase the level of H_2O_2 in liver cells, but in the higher concentration (3000 µg/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[17]. 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 oxidizied to Fe^{3+} by oxygen to form radical superoxid. Then, radical superoxide reacts with hydrogen to form H_2O_2. Furthermore, H_2O_2 could reacts again with Fe^{2+} and radical hydroxyl is produced[7,18-20].
- Haber-Weiss reaction. Superoxide and H_2O_2 that produced during fenton reaction could react to form hydroxyl radical which promote another reaction to form another ROS, etc[21].

H_2O_2 which produced by Fe can be use by MPO to oxidizied 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 by the presence of Fe, CI still increased in the liver cells. This result suggest that Fe still induced chlorinative stress. This result is similar to Setiawan *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 H_2O_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 siginficantly in liver cells especially when Fe level is excessive. This results also similar with Setiawan *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.

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