

Oxidative Stress and Protein Carbonylation as a New Tool to Assess Redox Status in Liver Toxicity Induced by Iron

Nia Kania¹, Iskandar Thalib², Eko Suhartono³

¹Research Center for Toxicology, Cancer, and Degenerative Disease, Department of Pathology, Ulin Genral Hospital/Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia

²Research Unit of Mutiara Bunda Mother and Child Hospital, Martapura of South Kalimantan, Indonesia

³Department of Medical Chemistry/Biochemistry, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia

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ABSTRACT

The objectives of this study were to determine the effect of iron (Fe) on Oxidative Stress Index (OSI) and Protein Carbonylation (PC), and their correlation in liver homogenate in vitro. In this experiment, a liver sample was taken from male rats (*Rattus norvegicus*). Samples then homogenized and divided into four groups with; T0 served as control which contain liver homogenate only; T1 which contains liver homogenate+30 µg/dl of ferrous sulphate (FeSO₄); T2 which contains liver homogenate+ 300 µg/dl of FeSO₄; and T3 which contains liver homogenate+3000 µg/dl of FeSO₄. After treatment, liver Total Oxidant (TO), Total Antioxidant (TA), OSI and PC level were estimated. The results revealed that Fe exposure could significantly increased TO, OSI, and PC level, and decreased TA level in liver cells (P<0.05). The result also revealed that OSI that induced by Fe have a strong correlation with PC (r=0,9388). These results indicated that Fe exposure induce OS which can be seen from the increasing of TO and OSI levels, and the decreasing of TA. Also, the results indicated that Fe exposure induced PC which related to Oxidative Stress (OS).

Keywords: Coal Dust, Iron, Oxidative Stress, Protein Carbonylation

INTRODUCTION

Indonesia is blessed with abundant mineral resources¹. One of the world's most plentiful mineral energy that can be found in Indonesia is coal². In Indonesia the coal resources are plenty, particularly in several area including South Kalimantan³. It is well documented that the coal usage raises a number of environmental challenges. One of the important environmental problem caused by coal mining is air pollution⁴.

In each process of coal mining, a great amount of dust is produced⁵. Coal mine dust is a mixture that contains more than 50 substances⁶. According to Setiawan et al⁷. study, the coal dust contained several metal with the highest metal level is iron (Fe). Fe is a necessary chemical element, involved in a wide spectrum of fundamental biological processes. However, Fe have several adverse effects in excessive amounts⁸. Several studies reported that exposure to excess Fe doses may lead to liver cell damage, mutation, and malignant transformations⁹.

Fe toxicity is developed through the production of reactive oxygen species (ROS)¹⁰. Our previous study showed that Fe could induced the formation of ROS, such as hydrogen peroxide¹¹. Fe may contribute significantly to the generation of ROS via Fenton and Haber-Weiss reaction. Increase the amount of ROS could promote lipid peroxidation and changes in antioxidant activity¹²⁻¹³. This condition could shift the balance of oxidative status, known as oxidative stress (OS)¹⁴.

Recently, OS not only evaluated by analyze the level of oxidants and antioxidants, but also analyze through a proportion or ratio. This ratio was first proposed by Sharma et al.¹⁵ and known as OS index (OSI)¹⁶. OSI is a ratio between Total Oxidants Status (TOS) and Total Antioxidant Status (TAS)¹⁷⁻¹⁸. Generally, TOS and TAS was measured by the method of Erel. This present study used a different approach. OSI is calculated by measuring the ratio between H₂O₂ level (Total Oxidant (TO)) and the total activity of SOD, CAT, and Pox (Total Antioxidant (TA)). OS could promote several biomolecule modifications. Among a wide range of OS-derived modifications, Protein Carbonylation (PC) is known to be a major hallmark of OS¹⁹⁻²⁰. PC is a type of protein oxidation that can be promoted by OS condition. ROS during OS condition yields highly reactive carbonyl derivatives resulting either from oxidation of the side chains of lysine, arginine, proline, and threonine residues – particularly via metal-catalysed oxidation-or from the cleavage of peptide bonds by the α -amidation pathway or by oxidation of glutamyl residues. This chain reaction leading to ageneration of some protein carbonyls²⁰. PC is the most widely used biomarker for oxidative damage to proteins, and reflects cellular damage induced by multiple forms of ROS²¹. Considering the Fe is related to OS status and OS related to PC, this present study is aimed to measured the OSI, PC level, and correlation between those two parameters in liver homogenate.

MATERIAL AND METHODS

Animals and Homogenate Preparation

Male rats (*Rattus norvegicus*) 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 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 TOS, TAS, OSI and PC level were estimated.

TO analysis

The TO was analyze through the measurement of H₂O₂. 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 measured at λ=505 nm, using the supernatant of each solution. H₂O₂ level was expressed as µM²²⁻²³.

TA analysis

TA is simply the sum of the measurable antioxidants. The measurable antioxidants were SOD, CAT, and Pox. SOD was measured according to the method that previously described by Misra and Fridovich¹³. CAT activity was measured by the method of Aebi²⁴. Pox activity was measured by the method of Pruitt²⁵.

Oxidative stress index analysis

The OSI was a ratio between TO and TA. It was calculated following to equation:

$$\text{OSI } (\mu\text{M/unit}) = \frac{\text{TO } (\mu\text{M})}{\text{TA (unit)}}$$

Protein carbonyl level analysis

PC was calculated by measuring the total protein carbonyl content. The total protein carbonyl content was determined by colorimetric method. The liver

homogenate (0.5ml) was pipetted into 1.5 ml centrifuge tube and 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added and allowed to stand at room temperature for 1 hour, with vortexing every 10-15 minutes. Then, 0.5ml of 20% Trichloroacetic acid was added followed by centrifugation. The supernatant was discarded and the pellets was washed 3 times with 1 ml ethanol-ethyl acetate (1:1) to remove free reagent. The obtained precipitated protein was redissolved in 0.6 ml guanidine solution. Carbonyl content was calculated from maximum absorbance (390nm)²².

Statistical analysis

The results were expressed as mean±SE for three replicates. Significance of mean differences of all TO, TA, OSI, and PC between treatment and control groups were statistically compared using one-way Analysis of Variance (ANOVA) or Kruskal-Wallis test and followed by a post hoc Tukey's Honestly Significant Difference (HSD) or Mann-Whitney test for multiple range test. To determine the relationship between OS and PC, OSI was correlated to PC. The linear correlation regression was used to analyse the relationship between those variables and the correlation coefficient (r) was derived to measured the strength of association between those two parameters. Significance was set at P<0.05. The software used for the data analysis were the Statistical Package for the Social Sciences (SPSS) version 16.0 and Microsoft Excell 2010 for Windows Vista.

RESULTS

In this present study, OS status in liver homogenate induced by iron was evaluated by measuring OSI. To obtained the level of OSI, TO and TA must calculated. The results shows in figure 1 and 2. According to figure 1 and 2, Fe exposure seems to increase the level of TO and decrease the level of TA. ANOVA test results shows that Fe exposure cause a significant increase in TO level and significant decrease in TA level (p<0.05). Post Hoc Tukey HSD test results show that there are significant differences in TO level between between T0 and T3, and T1 and T3 group, and in TA there are significant differences between group of treatments except between T2 and T3 group (table 1).

After determine the TO and TA level, the level of OSI can be obtained. The results shows in figure 3. After the administration Fe, OSI level seems to be increase compared to the controls (Figure 3). There was a dose dependent increase in the OSI level in the liver tissues. ANOVA test results shows that Fe exposure cause a significant increase in OSI level (p<0.05). Post Hoc Tukey HSD test results show that there are significant differences between T0 and T3, and T1 and T3 group (table 1).

The effect of different dose of Fe on PC level is presented in figure 4. It was observed that the PC level increase in all Fe treatment groups as compared to control group. The increase in PC level were in a dose dependent manner. However, statistical analysis test results shows that the decreasing of MPO activity were not statistically significant (Kruskal-Wallis test, P>0.05). Figure 5 shows

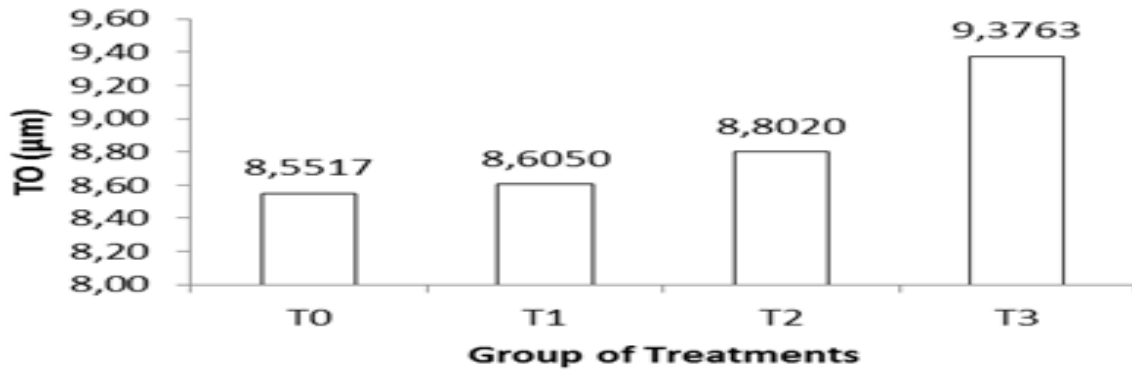


Figure 1: Comparison of TO level between group of treatments

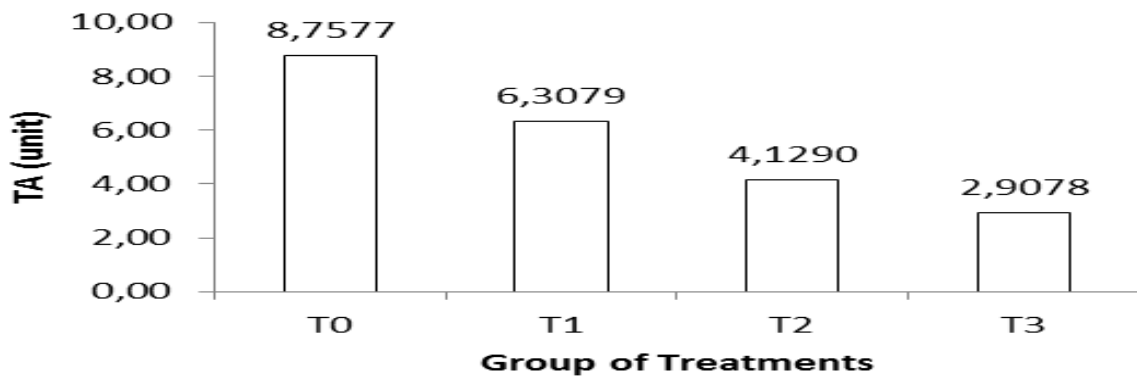


Figure 2: Comparison of TA between group of treatments

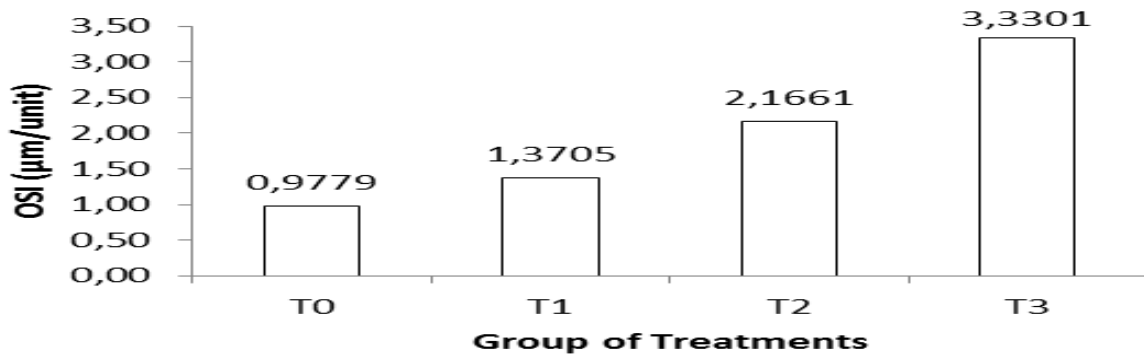


Figure 3: Comparison of OSI level between group of treatments

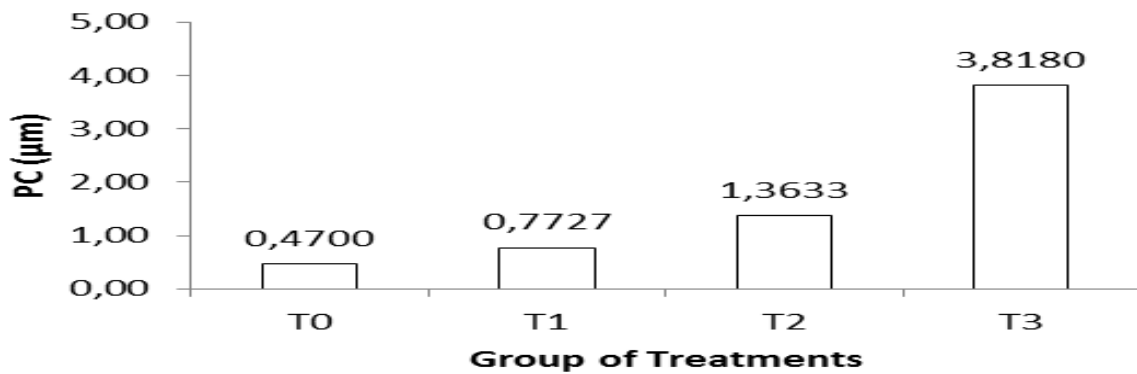


Figure 4: Comparison of PC level between group of treatments

the correlation between the level of OSI and the level of PC in liver homogenate induced by Fe with different

concentrations. OSI level was strong positively correlated with PC level ($r = 0,938$).

Table 1: Post Hoc Tukey HSD test results of TO, TA, and OSI level in the different group of treatments

Group Comparison	TO		TA		OSI	
	p-value	Interpretation	p-value	Interpretation	p-value	Interpretation
T0-T1	0.727	NS	0.001	S	0.285	NS
T0-T2	0.128	NS	0.000	S	0.008	NS
T0-T3	0.001	S	0.000	S	0.000	S
T1-T2	0.218	NS	0.001	S	0.049	NS
T1-T3	0.001	S	0.000	S	0.000	S
T2-T3	0.005	NS	0.029	NS	0.009	NS

TO: Total Oxidant; TA: Total Antioxidant; OSI: Oxidative Stress Index S: Significant; NS: Non-Significant; T0: control group; T1: treatment with 30 µg/dl; T2: treatment with 300 µg/dl; T3: treatment with 3000 µg/dl. Results presented as mean±SD. p-Values were calculated using the One Way ANOVA followed by Post Hoc Tukey HSD or Kruskal-Wallis test and followed by Mann-Whitney test; $p < 0.05$ was considered statistically significant.

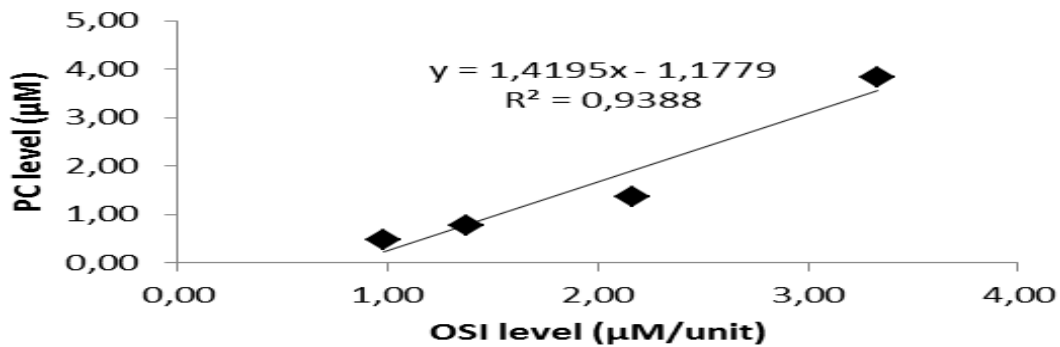


Figure 5: Correlation between OSI level and PC level in liver homogenate after iron exposure.

DISCUSSION

Fe is crucial to biologic functions, including respiration, energy production, DNA synthesis, and cell proliferation. The human body has evolved to conserve Fe in several ways, including the recycling of Fe after the breakdown of red cells and the retention of Fe in the absence of an excretion mechanism²⁶. However, although Fe is vital for so many metabolic reactions, free Fe is toxic to tissue, damaging cellular membranes, proteins, and DNA²⁷. The toxicity of Fe was known related to the property to generate ROS. Results of this present study revealed an oxidative stress in liver homogenate exposed to Fe in different concentration. It can be seen from the level of TO, TA, and OSI. However, to best of our knowledge, there have been no investigations of association between Fe exposure with OS in liver cells homogenate with this parameters. However, several reports have documented a relationship between excessive Fe diets and OS in human and animal model²⁸⁻²⁹.

The level of TO was significantly increased especially if the liver exposed to Fe in higher concentrations (3000 µg/dl). This is because the measurement of TO only measured H_2O_2 , and not measured the other ROS such as, radical superoxide and radical hydroxyl. It is well known that Fe induced the formation of ROS via both Fenton and Haber-Weiss reaction. The ROS that produced during this two reactions were H_2O_2 , radical superoxide, and radical hydroxyl³⁰.

Besides as a result of increased levels of ROS, OS can occurred as a result of reduced physiological activity of antioxidant defenses against ROS³¹. It was supported by the result of this present study. The result revealed that Fe

exposure in all concentrations could significantly decreased the TA in liver homogenate. From this results, we can also assumed that Fe exposure affect all enzymatic antioxidant. From this point of view, we can also assumed that Fe not only increased the level of H_2O_2 , but also increased another ROS such as radical superoxide and hydroxyl radical. Again, this result is in line with TOS level result that mentioned earlier. Recently, OS is evaluated not only by measuring the levels of oxidants and antioxidants. OS can also evaluated by measuring the ratio between them. This first approach was made in human medicine by Sharma et al.¹⁵ In this study we used that approach but with slight modification. The result revealed that Fe exposure increased OSI. This increasing indicated that the increasing risk of OS because of the increase in ROS production or defensive antioxidant consumption¹⁷.

OS elicited oxidative damage to several biomolecules, including protein³². Protein damage by OS can be evaluated with several marker, such as PC. PC is a type of protein oxidation that can be promoted by ROS and PC is the most widely used biomarker for oxidative damage to protein²⁰. It also in line with this results of this present study. According to the result, Fe exposure increase OS as can be seen by the increasing of OSI. OS then induced the protein damage that can be seen by the increasing of PC level and strong correlation between OSI and PC level in liver cells. However, as far as we know, there have been no investigations of association between OS and PC and their correlation induced by Fe in liver cells homogenate.

In conclusion, the present study demonstrated that Fe induced OS and protein damage as can be seen from the level of OSI and PC in liver cells. Also, the present study demonstrated that OS induced by Fe, related to protein damage in liver cells.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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