Oxidative and Chlorinative Stress Biomarkers in Liver Cells of Rats Exposed to Cyanide *In-Vitro*

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ABSTRACT

Cyanide is one of the rapid acting poisons. Exposure to cyanide can cause several health problems, including liver cells damaged. However, the mechanism of liver cells damage by cyanide still not clear. In this present study, we proposed that cyanide could induced liver cells damaged via oxidative and chlorinative stress pathway. Thus, our study aimed to investigated the effect of cyanide on liver cells via oxidative and chlorinative stress pathway by measuring the levels of hydrogen peroxide (H_2O_2) malondialdehyde (MDA), Advanced Oxidation Protein Products (AOPPs), the myeloperoxidase (MPO) acivity, and the chlorinative index (CI). The results revealed that cyanide exposure positively correlated with H_2O_2, MDA, AOPPs level, and CI, and negatively correlated with MPO activity in liver cells homogenate. These results indicated that cyanide exposure induce liver cells damage through the oxidative and chlorinative stress pathway which can be seen from the correlation between cyanide exposure and the levels of H_2O_2, MDA, and AOPPs, the activity of MPO, and the CI.

Keywords: Chlorinative Stress, Cyanide, Oxidative Stress

INTRODUCTION

Cyanide is one of the most deadly rapid-acting poisons. Cyanide toxicity is generally considered to be a rare form of poisoning[1]. Altough less frequent than another poisoning, cyanide posses significant physical harm, due to its rapid and potentially fatal adverse effects[2]. Recently, this compound was popular especially in Indonesia. It is because the case of Australian student with Indonesia citizenship, named Jesica Kumolo Wongso, who is a murder suspect in the case of cyanide-lace coffee in Indonesia[3]. Cyanide is one of the most toxic chemical substances on Earth. Cyanide may be produced by toxic gases during the pyrolysis of plastic or nitrile-based polymer fibres, by extracts of plants containing cyanogenic glycosides (e.g. cassava) or from industrial waste (e.g. electroplating)[4]. The extreme toxicity of cyanide arises from its readiness to react with other elements and hence interfere with normal biological processes[5]. Both acute and chronic exposures of cyanide can cause severe poisoning in human and animals[6]. Experimental studies on different animal species have shown that prolonged sublethal cyanide exposure can induce pathologic effects on various organs, including liver[4,7]. The mechanisms by which cyanide exert its effects to liver have not all been fully elucidated. It is also widely accepeted that cyanide itself stimulates production of intracellular reactive oxygen species (ROS). It is thought that cyanide exposure causes rising of oxidative stress by glutathione (GSH) depletion and inhibition of a series of antioxidant enzymes. Also, It is reported that cyanide exposure increases levels of ROS and reactive nitrogen species (RNS) and the amount of lipid peroxidation[8]. The increasing of ROS by cyanide will increase the concentration of hydrogen peroxide (H_2O_2) to form radical hydroxyl with the presence of transition metal[9-11]. These ROS can randomly react with lipids, proteins and nucleic acids causing oxidative stress and damage in these macromolecules. When ROS target lipids, they can initiate the lipid peroxidation process, a chain reaction that produces multiple breakdown molecules[12]. Among these multiple breakdown molecules, malondialdehyde (MDA) is the one of the most well studied markers of lipid peroxidation[13-15]. However, the increasing concentration of H_2O_2 also have another effect. H_2O_2 can be used by myeloperoxidase enzyme (MPO) to produce another oxidants, known as hypochlorous acid (HOCl)[9]. HOCl can promote a further reaction resulted in a condition known as chlorinative stress[16-19]. According our previous study, chlorinative stress can be measured by calculating both the ratio between H_2O_2 level and MPO activity, known as chlorinative index (CI) and the level of Advance Oxidation Protein Products (AOPPs)[19-21]. Based on the fact that oxidative and chlorinative stress may be involve in cyanide toxicity. Thus, we hypothesized that, cyanide-induced liver cells damage may follow the oxidative and

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chlorinative stress pathway; and we designed the present study to explore this hypothesis by measured several parameters, such as H_2O_2, MDA and AOPPs levels, MPO activity, and CI value in liver cells homogenate.

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 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
The present study was a experimental study design to examine the liver celles damage induced by cyanide through the oxidative and chlorinative stress pathway. Liver cells will exposed to different concentration of cyanide. The cyanide concentrations were 0 mg/l, 0.5 mg/l, 1 mg/l, and 2 mg/l. Each solution then incubated at 37°C for 3 hour. After incubation, liver H_2O_2, MDA, 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[22].
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 1.56×105 mol/cm. The MDA concentration expressed in μmol MDA.As a standard solution we used commercially MDA[22].
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[23].
Chlorinative index analysis
Chlorinative index was a ratio between H_2O_2 level and MPO activity. Chlorinative index was calculated following to equation[20].

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

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[24].
Statistical analysis
The results were expressed as mean±SE for six replicates. The data was analysed between each parameter level and cyanide concentration. For analyzing the data, microsoft excell 2010 was used and was examined by simple correlation regression.

RESULTS AND DISCUSSION
In this present study, to investigate the involvement of oxidative stress in cyanide-induce liver cells damage, the level of liver H2O2 and MDA was investigated. The results shows in figure 1 and 2. Cyanide exposure caused the increasing of liver H_2O_2 and MDA levels. From figure 1 and 2, we can see that the increasing of cyanide concentration can increase the level of both H_2O_2 and MDA. Cyanide concentration was strong positively correlated with liver H_2O_2 and MDA (rH2O2= 0,906; rMDA= 0,992). The results of this present study shows that cyanide have a strong possitive correlation with liver H_2O_2 and MDA level. It means the increasing level of cyanide in liver will increase both H_2O_2 and MDA level. The results in this study confirm that cyanide exposure increase oxidative stress and lipid peroxidation in the brain and kidney of mice and rats[25,26]. It is been widely accepted that cyanide-induced cellular oxidative stress, i.e., increase of superoxide anions, lipid peroxide, hydroxide radicals, hydrogen peroxide and others, appears to arise through multiple pathways. At the cellular level, cyanide produces chemical hypoxia by
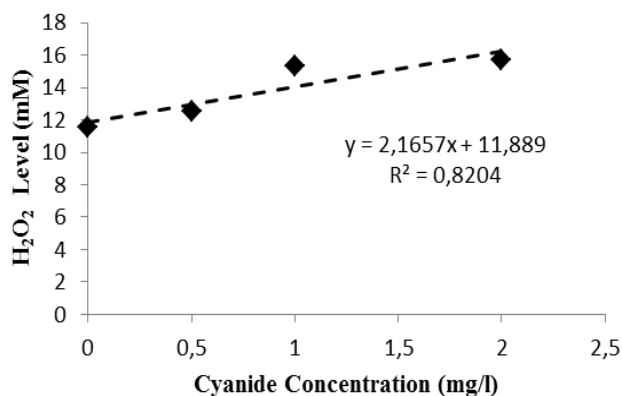
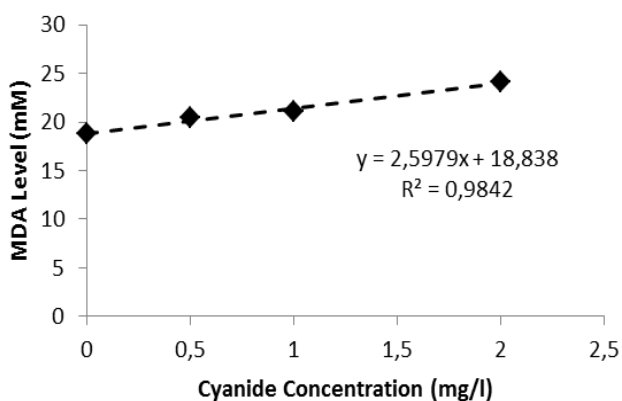Figure 1: Correlation between cyanide concentrations and liver H_2O_2 level

Figure 3: Correlation between cyanide concentrations and liver MPO activity

Figure 2: Correlation between cyanide concentrations and liver MDA level

Figure 4: Correlation between cyanide concentrations and liver AOPPs level

inhibiting cytochrome c oxidase in complex IV of the mitochondrial oxidative phosphorylation chain to markedly reduce ATP[27]. Furthermore, it can block the flow of electrons through complex IV to prevent oxidative metabolism and enhance ROS generation at complex III[26]. Another mechanism that cyaninde-indeuced celluar oxidative stress is inhibits antioxidant enzymes like GPx and depletes non-enzymatic antioxidants like intracellular GSH[28]. It was also reported that cyanide causes an increase in lipid peroxidation and a decrease in GSH levels, and GPx, SOD, and CAT activities in liver of rats[29]. Okolie and Iroanya[30] reported inhibition of SOD and CAT in liver, kidney and lung tissues of the cyanide treated rabbits. Such enzymes containing metal porphyrins as peroxidase, CAT and xanthine oxidase are inhibited strongly by cyanide[8]. MPO activity in the liver that received different doses of cyanide are presented in the Figures 3. After the administration cyanide, MPO activity seems to be decreased (Figure 2). There was a dose dependent decrease in MPO activity in the liver tissues. Cyanide concentration was strong negatively correlated with liver MPO activity. MPO is a member of peroxidase. MPO is a heterodimeric, cationic and glycosylated haem enzyme[31]. The basic reaction of MPO is decompose H_2O_2 into water and oxygen[32]. In the other hand, in several conditions, MPO also decompose the oxidation of chloride to HOCl[33]. The results of this present study shows that
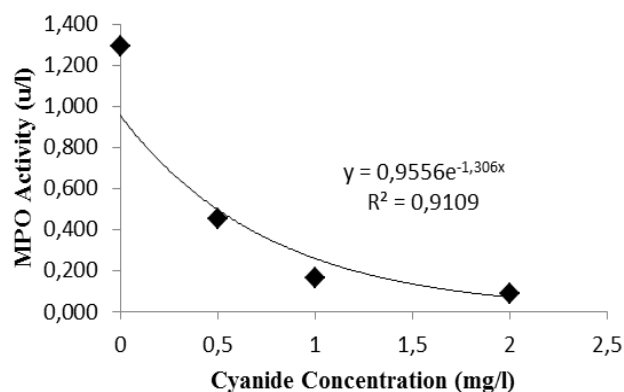

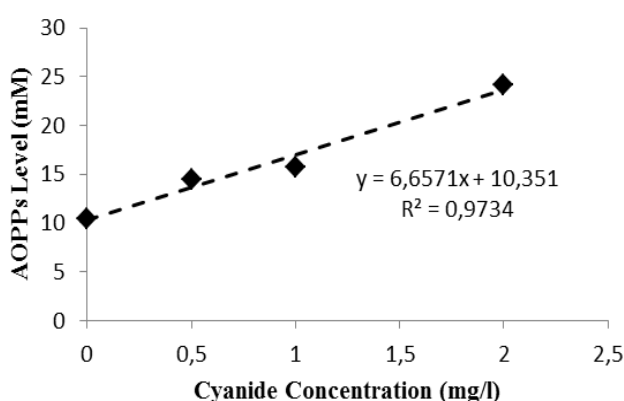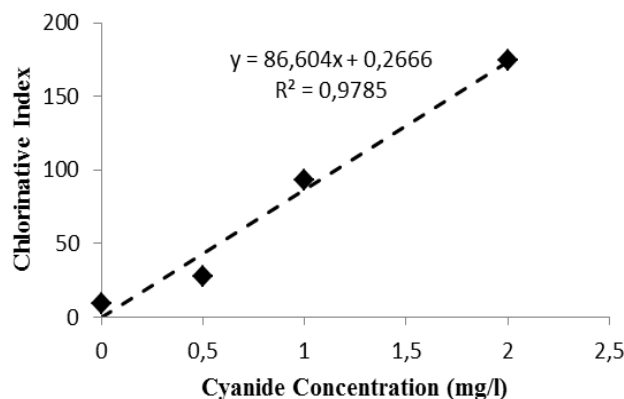Figure 5: Correlation between cyanide concentrations and liver CI

cyanide interrupt the MPO activity. The interruption of MPO activity by cyanide may be cause by the effcet of cyanide to induce a chlorinative stress condition. It is based on our several results study. Based on our several results, the presence of H_2O_2 which in this study induced by cyanide, can activated MPO to promote a further reaction to form HOCl and induce a condition, known as chlorinative stress[17,19-20]. To prove it, in this present study we measured another two parameters that indicate chlorinative stress, such as AOPPs and CI (figure 4 and 5). From figure 4 and 5, we can see that cyanide exposure in liver is strong posstively correlated with AOPPs level and CI. This two parameters indicated that cyanide

exposure induce chlorinative stress in liver. AOPPs is a novel markers of oxidant-mediated protein damage by the reaction of plasma proteins with chlorinated oxidants[34]. Since AOPPs formed by chlorinated oxidants, it can also used as chlorinative stress condition[21]. Besides AOPPs, chlorinative stress can also be seen by CI. It is based on previous research by Hartoyo[19] which investigated the involvement of chlorinative stress in dengue hemorrhagic fever patients. Also, another previous study by Marisa[17] and Kania[20] in anti-tuberculosis medicine and iron induced liver toxixicty through the chlorinative stress pathway. In conclusion, the present study demonstrated that cyanide induced oxidative and chlorinative stress as can be seen from the level of H_2O_2, MDA, and AOPPs, the activity of MPO, and the CI in liver cells.

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

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