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Research Article

Hepatotoxicity Effect of Rifampicin and Isoniazid via Chlorinative Stress Pathway Mechanism *In-vitro*

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ABSTRACT
In this present study, we try to investigate the hepatotoxicity effect of RIF and INH vis chlorinative stress pathway *in vitro*, by measured catalase (CAT) kinetic parameters and Advanced Oxidation Protein Products (AOPPs) level. In this experiment, a liver sample was taken from male rats (*Rattus novergicus*). Sample the homogenized and divided into three groups with; T0 served as control which contains liver, T1 which contains liver homogenate + 450 mg Rifampicin (RIF); and T2 which contains liver + 300 mg Isoniazid (INH). For CAT kinetic parameters analysis (Km, Vmax, and kcat), in each solution was added hydrogen peroxide (H_2O_2) with different concentrations. Solutions then incubated at 37ºC for 1 hours and then was prepared for kinetic parameter analysis. The AOPPs level was analysed using spectrophotometric methods. The results showed that both RIF and INH treatments could decrease the affinity of H_2O_2-CAT complex and increase the turnover rate of the reaction which expressed by the higher Km, Vmax, and kcat values. Also, the results showed that both RIF and INH significantly increased the level of AOPPs in liver homogenate. From this results, it can be concluded that both RIF and INH-induced hepatotoxicity via chlorinative stress pathway by disrupting the CAT activity and increased the AOPPs level.

Key Words Chlorinative Stress, Hepatotoxicity, Isoniazid, Rifampicin

INTRODUCTION

Tuberculosis (TB) is an infectious disease of epidemic proportions caused by the bacillus *Mycobacterium tuberculosis*[1]. Each year, TB killing nearly 1.6 million people, mostly in developing countries including Indonesia[2-3]. According to the World Health Organization (WHO), there are about 450,000 (95% confidence interval, 370,000 to 540,000) newly diagnosed TB patients each year in Indonesia[4]. Current treatment of TB is a combination therapy consisting isoniazid (INH), rifampicin (RIF), pyrazinamide (PYZ), and ethambutol (ETM)[5]. These drugs have a potential adverse effect including hepatotoxicity, especially for INH and RIF[6]. The frequency of hepatotoxicity varies from 2 to 28% in different populations and occurs even when the drug has been given at the recommended doses[5]. The exact mechanism responsible for liver injury caused by INH dan RIF is not clear. Several studies indicated that oxidative stress may involve in INH and RIF induce liver damaged[6]. Oxidative stress is a shift in the balance between oxidants, such as radical superoxide, hydrogen peroxide, hydroxyl radical, etc., and antioxidants, such as superoxide dismutase (SOD), peroxidases, and catalase (CAT)[7,8]. Results of Thalla *et al* study suggest that RIF and INH could decrease the activity of several enzymatic antioxidants including CAT. As a consequence of CAT decreasing activity, the level of hydrogen peroxide is increased[9]. It is well known that hydrogen peroxide is a substrate for myeloperoxidase (MPO). The presence of hydrogen peroxide can activated MPO which could promote further reaction resulting in the formation of hypochlorous acid (HOCl)[10]. HOCl is a powerful oxidizing agent which is known to cause a condition known as chlorinative stress[11,12]. The pathological consequence of chlorinative stress is damage to macromolecules such as protein[13]. Proteins are elective targets of chlorinative stress, leading to cross-linking and aggregation products known as Advanced Oxidation Protein Products (AOPPs). AOPPs are dityrosine-containing and cross-linking protein products formed mainly by exposure of protein to HOCl[14-15]. AOPPs has known related to markers of monocyte activation, and may serve as mediators of inflammation[16-

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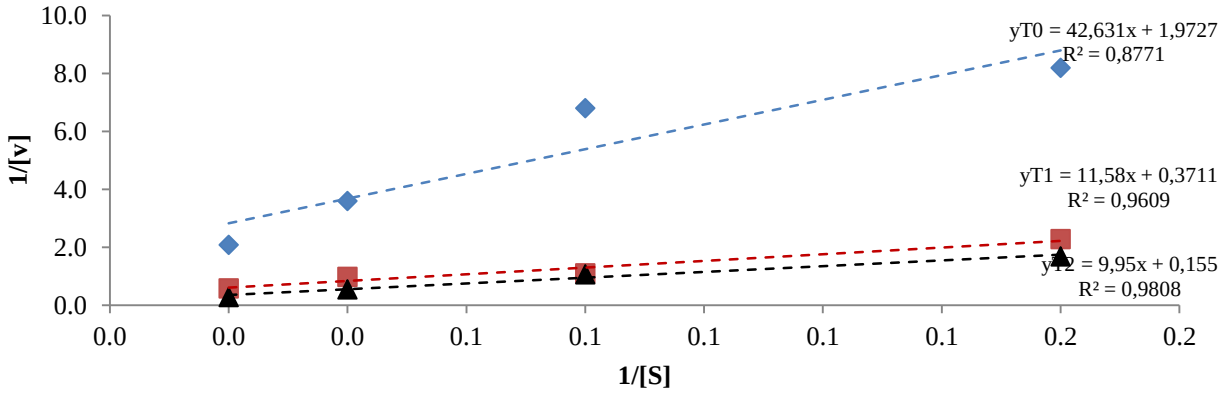

Figure 1: The Lineweaver-Burke plot for liver CAT in different groups of treatment. T0 represent the control group; T1 represent the RIF treatment group; and T2 represent the INH treatment group

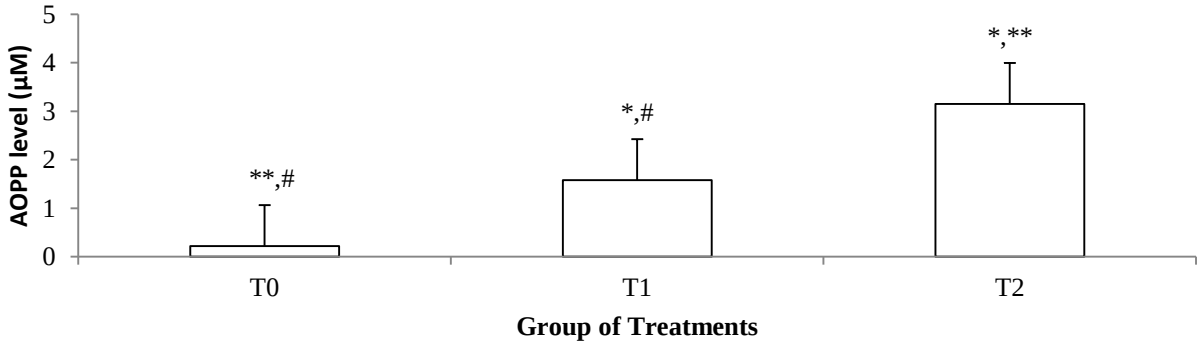

Figure 2: AOPPs level in different groups of treatment. T0 represent the control group; T1 represent the RIF treatment group; and T2 represent the INH treatment group. Values are mean±SEM. Statistically significant differences are indicated by * p<0,05 vs.control, ** p<0,05 vs. T1, # p<0,05 vs. T2 group.

[17]. To the best of our knowledge, in the literature survey, there is no scientific study on evaluation of INH and RIF induced hepatotoxicity via chlorinative stress pathway. Therefore, the present study was focused on evaluating the hepatoxicity effect of INH and RIF via chlorinative stress pathway by assessing kinetic parameters of CAT and AOPP level in liver homogenate.

MATERIAL AND METHODS

Samples collection: The liver samples were collected from 24 old male rats (*Rattus novergicus*) with 2-3-month-old and weighing 200-250 g. The rats were purchased from the Abadi Jaya farm at Yogyakarta, Indonesia, in healthy condition. Animals were the acclimatized to the laboratory conditions before samples collection. The rats were caged in quite temperature controlled room and had free access to water and standard rat diet. After the acclimatization period, the liver samples were taken by surgical procedure with ether as an anesthesia. Then the liver fixed in phosphate buffer at pH 7.0. The liver was ground to form a liquid. Subsequently, the solution was taken and centrifuged at 3500 rpm for 10 min and the top layer was taken and stored until it uses. All animals used and care was in compliance of the Ethics Commission of the Faculty of Medicine, University of Lambung Mangkurat, Banjarbaru, South Kalimantan, Indonesia.

Experimental models

Samples were divided into 3 groups (1 control group (T0) and 2 treatments groups (T1, and T2). Group 1: T0: liver homogenate only; Group 2: T1: liver homogenate + 450 mg of RIF; and Group 3: T2: liver homogenate + 300 mg of INH. Then, each of solutions was incubated at 37°C for 1 hour and undergo to catalase kinetic parameters and AOPP level analysis

Catalase kinetic parameter measurements

kinetic parameters were determined by using 4 different concentrations of the H_2O_2 as a substrate for catalase. The H_2O_2 concentrations were 50, 25, 12.5, and 6.25 mmol L^{-1}. The kinetic parameters Vmax and Km were determined using the Lineweaver-Burk version of the Michaelis-Menten equation[18], as follows:

$$\frac{1}{V} = \frac{km}{V\max} \cdot \frac{1}{[S]} + \frac{1}{V\max}$$

where V is the reaction velocity, Vmax is the maximum reaction velocity, Km is the Michaelis constant (the substrate concentration at half-maximal reaction velocity) and [S] is the substrate concentration. Using the Km and Vmax results that generated in the Michaelis-Menten equation, the catalytic constant or turnover number (k cat) was computed from Vmax using 118054.25 daltons (or g $mole^{-1}$) as the molecular weight of the enzyme.

AOPP level measurements

AOPP measurement were made by spectrophotometric methods as describe by Witko-Sarsat *et al*., with slight

Table 1: Kinetic parameters and coefficient correlation for liver catalase in different group of treatments

Group	Parameters			
	Vmax (sec^{-1})	Km (sec^{-1})	k cat ($\mu mol\ L^{-1}\ sec^{-1}$)	R
T0	0.507	21.611	2.072	0.937
T1	2.695	31.205	7.100	0.980
T2	6.451	64.185	13.443	0.980

T0: Control; T1: RIF; T2: INH

modification. Briefly, AOPP were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions that in the presence of potassium iodide at 340 nm. In test wells, 200 ml of plasma diluted 1/5 in phosphate buffer solution were placed on a 96-well microtiter plate and 20 ml of acetic acid was added. In standard wells, 10 ml of 1.16 mol kalium iodide was added to 200 ml of chloramine-T solution (0–100 mmol/l) followed by 20 ml of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm on the microplate reader against a blank containing 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/l. AOPP concentrations were expressed as μmol L^{-1} of chloramine-T equivalents[19].

Statistical analyses

Data are represented as mean±SEM. For comparing AOPPs level between group of treatments, one-way ANOVA followed by post hoc Tukey's Honestly Significant Difference (HSD) test was 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 17.0 and Microsoft Excell 2007 for Windows Vista.

RESULTS AND DISCUSSION

This present study which was undertaken to assess the effects of RIF, and INH on the kinetics parameters of CAT and AOPP level in liver homogenate. Kinetic parameters of liver CAT were determined from Lineweaver-Burke plot. This plot, along with the equation of the linear regression line and the correlation index of r^2 from each group of treatment can be seen in figure 1. From this plot, the values of Km, Vmax, and kcat for each group of treatment can be calculated. From the Lineweaver-Burke plot, the Vmax of the liver CAT in different group of treatments ranges from 0.507-6.451 sec^{-1} H_2O_2, while the Km values are within the range of 21.611-64.185 μmol/sec, and Kcat values are within the range of 2.072-13.433 (table 1). The

highest Vmax, Km, and Kcat values were found in the T0 group while the lowest was found in the T2 group. Table 1 also showed that the Vmax, Km and Kcat values are lower in all treatments groups (T1, and T2) compared to control. Results from the figure and table 1 suggest that RIF and INH applications decrease the values of Vmax, and Km, and increase the values of kcat. Vmax reflects the catalytic ability of an enzyme, Km reflects the affinity between enzyme and substrate, and kcat reflects the turnover rate of the reaction. Results of this present study indicated that both RIF and INH decrease the catalytic ability of CAT, the affinity between H_2O_2 and CAT and increase the turnover rate of the reaction. Results of this present study suggest that both RIF and INH could decrease the affinity between H_2O_2-CAT complex. It means the binding between H_2O_2 and CAT might be weak by the presence of both RIF and INH. The results also suggest that INH has a greater effect of decreasing the affinity than RIF. The increasing of kcat value also indicated that the turnover rate of the reaction was increased with the presence of both RIF and INH. Also, INH has a greater effect to increase the turnover rate of the reaction. The decreasing of affinity between H_2O_2-CAT complex and the increasing of the turnover rate will lead to increasing the concentration of H_2O_2 as a substrate and decrease the concentration of H_2O and O_2 as a product. From this point of view, both RIF and INH could increase the level of H_2O_2 and decreased te activity of CAT. Catalase was the first antioxidant enzyme to be discovered and characterized[20]. The main function of CAT is the removal of H_2O_2[21]. There are over 300 forms of catalase and the enzyme is found almost in all aerobically respiring organisms[22]. The highest concentrations in mammals are found in erythrocytes, liver and occasionally in the kidney and saliva[23]. Loss of CAT activity is associated with increased susceptibility to oxidative stress while overexpression studies suggest a role for the enzyme in longevity[22]. The results of this study clearly indicated that both RIF and INH-induced liver cell damaged, via oxidative pathway. The results also indicated that INH has a greater risk to induce liver damaged than RIF. The present study is in agreement with the earlier reports that RIF and INH-induced liver cell damaged via oxidative pathway[24,25]. Drug-induced liver damaged is the most commonly reported adverse reaction of RIF and INH[26]. RIF is a potent inducer of cytochrome P450 isoenzymes which mediates generation of toxic metabolites of drugs and their covalent binding to hepatic macromolecules while INH is believed to mediate hepatotoxicity through the production of toxic metabolites[6,26]. Those two basic mechanisms will increase the ROS formation such as H_2O_2 and inhibit the enzymatic antioxidants such as CAT[27]. The increasing of an H_2O_2 level and the decreasing of CAT activity by RIF and INH in liver homogenate could activate myeloperoxidase (MPO). MPO is a human peroxidase enzyme and a lysosomal protein stored in azurophilic granules of the neutrophil[28]. Using H_2O_2, MPO generates a wide array of reactive intermediates, such as HOCl[29]. The HOCl can react with plasma protein to form AOPPs. It can be seen from the results of this present study. The AOPPs level increase with the presence of both RIF and INH in liver homogenate (figure 2). Statistical analysis test results show that all groups of treatment significantly ($p<0.05$) increase

liver AOPPs concentration compare to control. The level of AOPPs in liver homogenate also significantly different between the group of treatments. Results of this present study indicated both RIF and INH induced the formation of AOPPs, with INH have a greater effect than RIF. Advanced oxidation protein products (AOPPs) were described by Witko-Sarsat et al[17,30]. AOPPs formed during oxidative (chlorinative) stress by reaction of plasma proteins with chlorinated oxidants[31]. AOPPs could be determined routinely using a simple protocol to investigate myeloperoxidase-induced protein modifications via chlorinative pathway[30,32]. In conclusion, the present study demonstrated that RIF and INH applications will disrupt the kinetic parameters (Km, Vmax, and kcat) of liver CAT. It seems both RIF and INH, increase the Km, Vmax, and kcat value of liver CAT. This indicated treatment with RIF and INH decreased the affinity between H_2O_2-CAT complex and increase the turnover number rate of the reaction. Also, the results of this present study indicated that treatment with RIF and INH increased the level of AOPPs in liver homogenate. Together these observations lead us to conclude that both RIF and INH could induce liver damaged via chlorinative stress pathway by inhibit the CAT activity and increased the level of AOPPs.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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REFERENCES

1. Saxena S, Karkhanis V, Joshi Jm. Tuberculosis prevention: an enigma worth untravelling. Indian Journal of Tuberculosis 2012; 59 (2): 65-67.
2. Kruk ME, Schwalbe NR, Aguiar CA. Timing of default from tuberculosis treatment: a systematic review. Tropical Medicine and International Health 2008; 13 (5): 703-712.
3. World Health Organization (WHO). A brief histroy of tuberculosis control in Indonesia. Geneva, WHO, 2009 (WHO/HTM/TB/2009.424).
4. Edine W. Tiemersma and Firdaus Hafidz. Costs faced by (multidrug resistant) tuberculosis patients during diagnosis and treatment. Netherlands, KNCV tuberculosis foundation, 2014.
5. Singh M, Sasi P, Rai G, Gupta VH, Amarapurkar D, Wangikar PP. Studies on toxicity of antitubercular drugs namely isoniazid, rifampicin, and pyrazinamide in an in vitro model of HepG2 cell line. Medical Chemistry Research 2010. doi: 10.1007/s00044-010-9405-3.
6. Bello B, Wudil AM, Atiku MK. Prevention of Oxidative Stress Caused by Anti-tubercular Drugs Using Aqueous Extracts of *Daucus carota* and *Moringa oleifera*. International Journal of Scientific and Research Publications 2013; 3 (10): 1-7.
7. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative Stress and Antioxidant Defense. WAO Journal 2012; 5: 9-19.
8. Al-Dalaen SM, Al-Qtaitat AI. Review article: oxidative stress versus antioxidants. American Journal of Bioscience and Bioengineering 2014; 2(5): 60-71.
9. Noor WF, Aprianti N, Saputra SR, Afifah B, Apriasari ML, Suhartono E. Oxidative stress on buccal mucosa wound in rats and rule of topical application of ethanolic extracts of mauli banana (*Musa acuminata*) stem. Journal of Tropical and Life Science 2015; 5 (2): 84-87.
10. Aksoy SN, Saygili WI, Bulbul F, Bahar A, Savas H, Virit O, Altindag A, Bayram A, Tarakcioglu. Myeloperoxidase enzyme levels and oxidative stress in bipolar disorders. African Journal of Biotechnology 2010; 9 (22): 3318-3323.
11. Yunanto A, Firdaus RT, Triawanti, Suhartono E. Advance Oxidation Protein Products (AOPPs) of newborn at risk of sepsis as novel parameter for early-onset neonatal sepsis. International Journal of Bioscience Biochemistry and Bioinformatics 2014; 4 (2): 90-93.
12. Joshi S, Peck AB, Khan SR. NADPH Oxidase as a therapeutic target for oxalate induced injury in kidneys. Oxidative Medicine and Cellular Longevity 2013; 2013 (462361): 1-18.
13. Locatelli F, Canaud B, Eckardt KU, Stenvinkel P, Wanner C, Zoccali C. Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. Neprology Dialysis Transplantation 2003; 18: 1272-1280.
14. Sebekova K, Klenovicsova K, Ferenczova J, Hedvig J, Podracka L, Heidland A. Advanced oxidation protein products and advanced glycation end products in children and adolescents with chronic renal insufficiency. Journal of Renal Nutrition 2012; 22 (1): 143-148.
15. Suhartono E, Triawanti, Leksono AS, Djati MS. The Role of Cadmium in Proteins Glycation by Glucose: Formation of Methylglyoxal and Hydrogen Peroxide in Vitro. Journal of Biomedical and Engineering 2014; 3 (1): 59-62.
16. Liu SX, Hou FF, Guo ZJ, Nagai R, Zhang WR, Liu ZQ, Zhou ZM, Zhou M, Xie D, Wang GB, Zhang X. Advanced oxidation protein products accelerate atherosclerosis through promoting oxidative stress and inflammation. Arteriosclerosis, Thrombosis, and Vascular Biology 2006; doi: 10.1161/01.ATV.0000214960.85469.68.
17. Suhartono E, Triawanti, Leksono AS, Djati MS. Oxidative Stress and Kidney Glycation in Rats Exposed Cadmium. IJCEA 2014. 5(6): 497-501.
18. Shang ZC, Zhang LL, Wu ZJ, Gong P, Li DP, Zhu P, Gao HJ. The activity and kinetic parameters of oxidoreductases in phaeozem in response to long-term fertiliser management. Journal of Soil Science and Plant Nutrition 2012; 12 (3): 605-615.
19. Husna AH, Ramadhani EA, Damayanti E, et al. The role formation of methylglyoal, carbonyl compound,

hydrogen peroxide and advanced oxidation protein product induced cadmium in ovarian rat. International Journal of Chemical Engineering and Applications 2014; 5: 319-323.
20. Mhamdi A, Queval G, Chaouch S, Vanderauwera S, Breusegem FV, Noctor G. Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. Journal of Experimental Botany 2010. doi:10.1093/jxb/erq282.
21. Yunanto A, Gunawan P, Iskandar, Suhartono E. Effect of antibiotic applications on salivary amlylase and catalase kinetic parameters on neonatal at risk of sepsis *in vitro*. International Journal of Toxicological and Pharmacological Research 2015; 7 (6). Article in Press.
22. Milton NGN. Homocysteine inhibits hydrogen peroxide breakdown by catalase. The Open Enzyme Inhibition Journal 2008; I: 34-41.
23. Kodydkova J, Vavrova L, Kocik M, Zak A. Human catalase, its polymorphisms, regulation and changes. Folia Biologica (Praha) 2014; 60: 153-167.
24. Tayal V, Kalra BS, Agarwal S, Khurana N, Gupta U. Hepatoprotective effect of tocopherol against isoniazid and rifampicin induced hepatotoxicity in albino rats. Indian Journal of Experimental Biology 2007; 45: 1031-1036.
25. Sankar M, Rajkumar J, Devi J. Hepatoprotective activity of hepatoplus on isonaizid and rifampicin induced hepatotoxicity in rats. Pakistan Journal of Pharmaceutical Sciences 2015; 28 (3): 983-990.
26. Tassaduq I, Butt SA, Hamid S. Protective effect of ascorbic acid on rifampicin induced hepatotoxicity in mice. Journal of Rawalpindi Medical College 2011; 15 (2): 102-103.
27. Schattenberg JM, Czaja MJ. Regulation of the effects of CYP2E1-induced oxidative stress by JNK signaling. Redox Biology 2014; 3: 7-15.
28. Tahboub YR, Fares MM. A predictive kinetic model for inhibitory effect of nitrite on myeloperoxidase catalytic activity towards oxidation of chloride. Journal of Biophysical Chemistry 2011; 2 (3): 202-207.
29. Heinecke JW, Goldberg IJ. Myeloperoxidase: a therapeutic target for preventing insulin resistance and the metabolic sequelae of obesity?. Diabetes 2014; 63: 4001-4003.
30. Demirbilek ME, Kilic N, Komurcu HF, Akin KO. Advanced oxidation protein products in aged with dementia. American Journalo of Immunology 2007; 3 (2): 52-55.
31. Li HY, Hou FF, Zhang X, Chen PY, Liu SX, Feng JX, Liu ZQ, Shan YX, Wang GB, Zhou ZM, Tian JW, Xie D. Advanced oxidation protein products accelerate renal fibrosis in a remnant kidney model. Journal of The American Society of Nephrology 2007; 18: 528-538.
32. Sung CC, Hsu YC, Chen CC, Lin YF, Wu CC. Oxidative stress and nucleic acid oxidation in patients with chronic kidney disease. Oxidative Medicine and Cellular Longevity 2013; 2013 (301982): 1-15.