

OXIDATIVE STRESS IN LIVER TISSUE OF RAT INDUCED BY CHRONIC SYSTEMIC HYPOXIA

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Abstract

Adaptation mechanism to hypoxia in living organisms increases reactive oxygen species (ROS) formation that could exceed the capacity of anti oxidant. Glutathione (GSH) in which highest concentration present in liver, plays an important role in maintaining the intracellular redox equilibrium and protect tissues from oxidative stress. The aim of this study was to observe tissue response of rat that was exposed to chronic systemic hypoxia by analyzing the oxidative stress in liver tissue. Twenty male Sprague-Dawley rats were induced by chronic systemic hypoxia by kept them in hypoxic chamber (10% O₂:90% N₂) for 1, 3, 7 and 14 day(s). All rats were sacrificed with ether anesthesia after hypoxia treatment. Liver tissues were analyzed using parameters of oxidative stress, malondialdehyde (MDA) with tBARS test, and endogenous antioxidant, glutathione reduced form (GSH). The study showed that chronic systemic hypoxia induction caused oxidative stress in liver tissue, which was shown by increased concentration of MDA in liver tissue (nmol/mg liver tissue). Concentration of MDA in liver tissue was increased significantly on day-1, day-3, day-7 and day-14 compared to control group (ANOVA, LSD, p<0.05). The differences between day-3, day-7 and day-14 was not significant. In contrast, liver GSH content (µg/mg liver protein) was progressively decreased significantly since day-1 of hypoxia until the end of experiment (ANOVA, LSD, p<0.05). Statistical analysis revealed that there is a strong correlation between MDA and GSH concentration in liver tissue (Pearson = - 0.993). It was concluded that oxidative stress present in liver tissue of rat induced by chronic systemic hypoxia.

Keywords: chronic systemic hypoxia, malondialdehyde (MDA), oxidative stress, reduced glutathione (GSH)

1. Introduction

Oxygen is very important for growth and development of multicellular organism. For this reason, multicellular organism, particularly human, was equipped with tools to bind oxygen in lung, transport it in a circulation and liberate it into the tissue. Oxygen is the terminal electron acceptor in oxidative phosphorylation in respiratory chain in mitochondria and substrate for almost 200 intracellular chemical reactions. Oxygen has functional as well as toxic effects.

Human, like many organisms, are sensitive to changes of oxygen concentration, such as hypoxia/anoxia. Susceptibility to hypoxic condition depend on the type of organism and tissue. For example, skeletal muscle is more tolerant to hypoxia compared to nerve tissue. Some organism can survive in environment with limited oxygen concentration. This condition is related to biochemical adaptation mechanisms which is made possible by metabolic control and metabolic regulation.

There are some biochemical adaptation mechanism as response to environment stress, not only hypoxia, but also as response to changes of environment temperature or ionic or water in the body. These adaptations are needed to maintain homeostasis, and hence maintain the physiological condition. The adaptation mechanisms involved the adaptation at protein/enzyme level or at the gene level.¹

One adaptation mechanism to hypoxia is the production of *hypoxia inducible factor-1α* (HIF-1α), by organisms, a protein which is a transcription factor due to its inhibition of degradation or increased its expression. Besides increasing the level of HIF, hypoxic condition also increase production and liberation of reactive oxygen species (ROS) in mitochondria and then regulated response to low level of oxygen concentration.²⁻⁴ Formation of ROS particularly present at complex III of electron transport chain where the ubisemiquinon radical donate its electron to oxygen forming anion superoxyde radical (O₂•-).^{5,6} Increased

ROS formation will trigger the signal transduction pathway to stabilized HIF.⁴ In hypoxic condition, ROS formation from this complex increased. If electron transport chain was inhibited in the proximal site with rotenon, ubiquinon was fully oxidized and ROS formation at site III was inhibited. Stabilization of HIF-1 α also present in normoxic condition induced by various cytokines and *growth factors*.^{7,8} Whether if HIF-1 activated or deactivated induced by ROS during hypoxia or activated in normoxic condition is still controversial.^{4,9,10}

During hypoxia, ROS formation is increased and lead to stress oxidative condition. If ROS formation exceed the capacity of antioxidant, ROS can react with macromolecules such as lipid, protein, and DNA which lead to cell dysfunction.^{2,11} Reaction of ROS with lipid membrane, rich in polyunsaturated fatty acids will cause lipid peroxidation. Malondialdehyde (MDA) is the end-product of lipid peroxydation and can be measured by thiobarbituric acid test (*TBARS test*).^{4,12,13}

Glutathione (GSH) is an important endogenous antioxidant which can scavenge ROS and maintain the intracellular redox equilibrium and protect tissues from oxidative stress. Glutathione scavenge the toxic effect of ROS catalyzed by glutathione peroxydase.^{4,14,15} There are two forms of glutathione, the active reduced form (GSH) and the inactive oxidized form (GSSG). The oxidized form of glutathione can be recycled into reduced form, catalyzed by glutathione reductase and supported by the NADPH from the hexose monophosphate shunt (*HMP shunt*).¹⁵

Liver is an organ with highest concentration of GSH compared to other tissues. High concentration of GSH also supported by the high activity of HMP shunt in liver tissue.^{12,15}

The aim of this study is to observe the relationship of chronic systemic hypoxia and oxidative stress in liver tissue using parameters of oxidative stress (e.g. malondialdehyde, MDA) and glutathione reduced form (GSH). Concentration of MDA and GSH were measured by Wills and Ellman methods respectively.

2. Methods

Materials. Reagents for MDA determination are tetraethoxypropane solution as standard solution, 20% trichloroacetic acid solution (TCA), and 0.67% thiobarbituric acid solution (TBA); reagents for GSH determination are glutathione standard solution, 5% trichloroacetic acid (TCA), 0.1M, pH 7, phosphate buffer saline (PBS), and dithiobisnitrobenzene (DTNB) solution. Reagent for protein determination is standard *bovine serum albumin* (BSA) solution.

Tools. Tools used in this experiment are deep-freezer -86°C, tissue homogenizer Potter-Elvehjem, refrigerated centrifuge, spectrophotometer, micropipette + microtip, microtube, balance scale.

Methods. Twenty five male Sprague-Dawley rats, 6-8 weeks olds, weighing 150-200 grams, were divided into 5 groups of five rats, according to Federer's formula: $(t-1) (n-1) \geq 15$.¹⁵ Rats were kept in Animal House of Department of Biochemistry and Molecular Biology Faculty of Medicine University of Indonesia. The treatment groups were placed in hypoxic chamber containing mix gas of 10% O₂ : 90% N₂ for 1, 3, 7 and 14 days, while the control group was exposed to the normal environment. Rats were supplied with water and food *ad libitum*. At the end of the treatment, rats were sacrificed with ether anesthesia. Liver tissue were taken out, weighed and divided into aliquots and immediately kept at -86°C. All procedures were approved by the Ethic Committee of Center Research and Health Development, Ministry of Health Republic of Indonesia (BALITBANGKES) No. LB.032.02/KE/4783/08.

Tissue preparation. One hundred milligrams of liver tissue were homogenized using 1 mL 0.2 M phosphate buffer saline (pH 7.4) and 0.1 mM phenylmethylsulfonylfluoride in microtube with micropestle, followed by centrifugation 700 g for 10 minutes. Each supernatant was collected and stored at -80°C.

Determination of the concentration of lipid peroxidation product.¹² The concentration of lipid peroxidation product, MDA, was determined using the thiobarbituric acid according to Wills method. Two mL thiobarbituric acid was added to the 200 μ L supernatant and boiled for 10 minutes in water bath. Absorbance of the color was measured at λ 530 nm. Concentration of MDA in supernatant of liver homogenate was calculated using a series of standard solution (0; 0.625; 1.25; 2.5; 5.0 nmol/mL). MDA was expressed as nmol MDA.mg liver tissue.

Determination of endogenous non enzymatic antioxidant concentration.¹⁶ The concentration of endogenous non enzymatic antioxidant, GSH, was determined using the dithiobisnitrobenzoate (DTNB) solution. Two hundred fifty μ L liver homogenate was added into 8.9 mL 0.1 M, phosphate buffer saline (pH 7). Then, 5% TCA was added to precipitate the protein. After centrifugation, 50 μ L DTNB was added to the supernatant of liver homogenate and incubated for an hour. The producing color of the substance was measured at λ 412 nm. Glutathione concentration in liver tissue was calculated using the standard curve of GSH standard solution (0; 10; 20; 40;50; 100 mg/mL). The concentration of GSH in liver tissue was expressed as μ g GSH/mg liver protein.

Determination of protein concentration. The liver protein concentration was measured by spectrophotometry at λ 280 nm using a series of standard bovine serum albumin (BSA) from 50–500 $\mu\text{g/mL}$.

Statistical analysis. Data from MDA and GSH concentration in liver tissue between normoxic and hypoxic groups were analyzed using two ways ANOVA and LSD. Differences between groups was considered significant if $p < 0.05$. Data values were reported as the Mean \pm SEM.

3. Results and Discussion

Protein concentration in liver tissue. Standard curve of BSA solution is presented in Figure 1.

Protein concentration. Protein concentration in liver tissue of all hypoxic groups are not different from the control group as shown from Table 1.

MDA concentration in liver tissue. MDA concentration of liver tissue in all groups of rats exposed to chronic systemic hypoxia were significantly higher than the control group as shown in Table 2 ($p < 0.05$, ANOVA, LSD). But, the differences between the MDA concentration in day-3, day-7 and day-14 of hypoxia were not significant ($p > 0.05$).



Figure 1. Standard Curve of BSA Solution

Table 1. Protein Concentration in Liver Tissue of Rat Induced by Chronic Systemic Hypoxia (mg/mg Liver Tissue).

No	Control	Day-1	Day-3	Day-7	Day-14
1	0.522	0.569	0.595	0.750	0.687
2	0.850	0.557	0.602	0.612	0.690
3	0.652	0.674	0.603	0.648	0.642
4	0.688	0.593	0.617	0.598	0.662
5	0.535	0.637	0.800	0.721	0.694
Mean \pm	0.649	0.606	0.643	0.666	0.675
SEM	\pm	\pm	\pm	\pm	\pm
	0.119	0.044	0.078	0.060	0.020

All hypoxic groups are not different significantly from control group ($p > 0.05$)

Table 2. MDA Concentration in Liver Tissue of Rat Induced by Chronic Systemic Hypoxia (nmol/mg Liver Tissue)

No	Control	1-day hypoxia	3-day hypoxia	7-day hypoxia	14-day hypoxia
1	0.07	0.014	0.020	0.043	0.042
2	0.012	0.027	0.024	0.026	0.024
3	0.011	0.018	0.020	0.029	0.030
4	0.009	0.016	0.035	0.025	0.031
5	0.010	0.018	0.041	0.025	0.040
Mean	0.010	0.019*	0.028* [^]	0.030* [^]	0.033* [^]
\pm	\pm	\pm	\pm	\pm	\pm
SEM	0.002	0.005	0.010	0.008	0.007

* Significant ($p < 0.05$, ANOVA followed by LSD) compared to control

[^] Not significant ($p > 0.05$, ANOVA followed by LSD) within day-3, day-7 and day-14 treatment

Table 3. GSH Content in Liver Tissue of Rat-Induced by Systemic Hypoxia ($\mu\text{g/mg}$ Liver Protein)

No	Control	1-day hypoxia	3-day hypoxia	7-day hypoxia	14-day hypoxia
1	0.059	0.047	0.034	0.021	0.020
2	0.055	0.049	0.036	0.028	0.023
3	0.052	0.039	0.034	0.029	0.024
4	0.051	0.039	0.031	0.032	0.025
5	0.054	0.033	0.028	0.023	0.017
Mean	0.054	0.041*	0.033*	0.027*	0.022*
\pm	\pm	\pm	\pm	\pm	\pm
SEM	0.003	0.006	0.003	0.004	0.003

* Significantly different ($p < 0.05$, ANOVA followed by LSD test)

GSH concentration in liver tissue. The concentration of GSH in liver tissue of rats exposed to chronic systemic hypoxia were significantly lower than the control group as shown in Table 3 ($p < 0.05$, ANOVA test followed by LSD). The level of GSH was decreased progressively from the day-1 until the day-14 of hypoxia.

To characterize the role of oxidative stress in liver tissue of rats exposed to chronic systemic hypoxia, we studied the production of MDA, the product of lipid peroxidation, the depleting content of GSH and the relationship between MDA and GSH content during hypoxia. Liver injury from hypoxia resulted from absolute or relative oxygen deficiency. Initial changes during cell hypoxia involved the mitochondria. A lack of oxygen in the final acceptor electron in electron transport chain, increase the mitochondria's reduced state due to an increase of NADH NAD⁺ ratio and lower ATP production from ADP. In our present study, we demonstrated that the level of MDA in liver tissue had increased since day-1 of hypoxia (1.90 fold higher

compared to control), day-3, day-7 and at the end of experiment at the day-14 (2.80 fold, 3.00 fold and 3.3 fold respectively). The oxidative stress seemed to increase with the length of exposure to hypoxia and reach the maximum level at the day-14 although the differences between the day-3, day-7 and day-14 are not significant any longer.

The elevated MDA concentration is due to increased ROS formation in mitochondria as a consequence of hypoxia. The increase level of MDA in liver tissue also proved by Jun *et al.* which found that oxidative stress caused by exposure to intermittent hypoxia for 1 week resulted in a trend to an increase in MDA level in liver tissue, while in the aorta and heart, intermittent hypoxia did not affect the MDA level.¹⁸ Increased ROS formation from mitochondria will trigger the redox signaling cascade. ROS will activate signaling pathways and in turn activate transcription factor and expression of target gene.⁴ HIF-1 α is one of many target genes which is activated by ROS. Increased level of ROS during systemic hypoxia in this experiment is counter-balanced by antioxidant system in liver tissue.

We also demonstrated that the concentration of GSH fell progressively the day-1 of hypoxia (0.76 fold), day-3, day-7 and day-14 at the end of the experiment (0.60 fold, 0.49 fold, 0.40 fold respectively). The glutathione level is decreased since the early phase of hypoxia and continued until the end of observation of hypoxia on day-14. This condition is caused by decreased antioxidant capacity of the liver. Antioxidant capacity of the liver is maintained by glutathione in reduced form (GSH), enzymatic antioxidant such as glutathione peroxidase and glutathione reductase, which is supported by the HMP shunt pathway producing the NADPH.^{12,15} Although the liver is rich with antioxidant, but since GSH were used to counter-balance the increased formation of ROS during hypoxic condition, the GSH level was more depleted at the day-14 of hypoxia. Glutathione is an of endogenous antioxidant which protects tissues from oxidative stress. Glutathione is a substrate for glutathione peroxidase, the enzyme that reduces hydroperoxides and organic peroxides, including lipid peroxide.¹⁴ Depletion of GSH due to oxidative stress was also proved by Samarsinghe *et al.* which found that oxidative stress due to hypoxia followed by reoxygenation showed fell of cellular GSH level to 37 % compared to the normoxic control group ($p < 0.001$).¹⁹

Increased formation of MDA concentration and depletion of GSH concentration showed that oxidative stress present in liver tissue of rat induced by systemic hypoxia. The comparison of MDA and GSH level was showed in Figure 2.

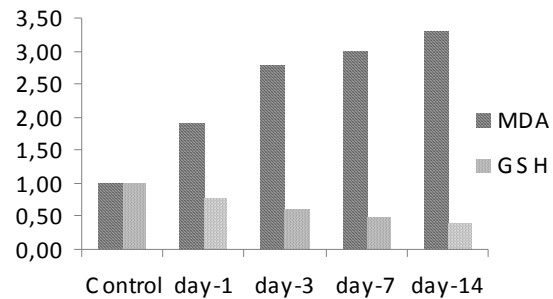


Figure 2. Comparison of MDA and GSH Concentration in Liver Tissue of Rat Induced by Chronic Systemic Hypoxia Normalized to Control Group

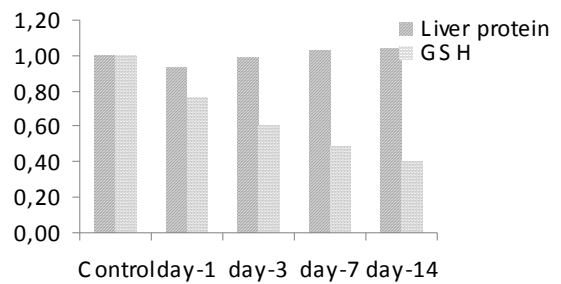


Figure 3. Comparison of Liver Protein and GSH Concentration in Rat Induced by Chronic Systemic Hypoxia Normalized to Control Group

It can be seen from that the relationship of liver GSH and MDA is inverted. The decrease of GSH is accompanied by the increase of MDA. Statistical analysis revealed that there is a strong negative correlation between both parameters (Pearson = - 0.993).

The concentration of GSH in liver tissue was expressed as $\mu\text{g}/\text{mg}$ liver protein. Although the GSH concentration in liver tissue had shown a significant decrease, but the concentration of liver protein between all groups are not significantly different ($p > 0.05$). Correlation analysis showed that there was a moderate negative correlation between the liver protein and GSH concentration (Pearson = -0.544). Chronic systemic hypoxia treatment did not have any influences on the liver protein concentration. It seems that during chronic hypoxia, protein synthesis in the liver was maintained to support vital liver functions, despite its depleted GSH concentration. Figure 3 shows the changes of liver protein and GSH concentration in all hypoxic groups which is normalized to control group.

4. Conclusion

The concentration of malondialdehyde, the end-product of lipid peroxidation, is increased in liver of rat induced by chronic systemic hypoxia. The concentration of glutathione, an endogenous antioxidant, is decreased in liver of rat induced by chronic systemic hypoxia. Oxidative stress present in rat liver tissue as a response to chronic systemic hypoxia.

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