CHRONIC HYPOXIC EXPOSURE INCREASED THE LEVEL OF MYOGLOBIN PROTEIN RATTUS NORVEGICUS KIDNEY
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ABSTRACT
Hypoxia is a lack of oxygen condition in tissues. In hypoxia, an animal’s body has to survive and regenerate its tissues. This can be done by increasing the myoglobin protein with serves to store tissues oxygen. This research aimed to find out if there was any effect of hypoxic exposure 90% N2 gas 90 percent to the level of myoglobin protein in a rat’s kidney. The research was conducted and employed an experiment method with Complete Random Design. The subjects of this research were twenty four rats that were put in a control group and a treatment group of hypoxic exposure for one day, three days, seven days, fourteen days and twenty one days. Four rats were put for each length of the exposure. Detection of myoglobin protein level was conducted in the kidney through a spectrophotometry and an immunodotblot technique. The result of this research showed that peak of myoglobin content increased in the treatment group fourteen and twenty one day than the control group.

Key words: myoglobin, kidney, Rattus norvegicus, spectrophotometry, immunodotblot, chronic hypoxic exposure.

INTRODUCTION
Animal’s body needs energy to do many activities. The cell process which generates ATP can be conducted aerobically or anaerobically (Nelson and Cox 2004, Halliwe and Gutteridge 2007). More ATP is generated in an aerobic process, which was 38 ATP, compared to the 2 ATP generated in an anaerobic process (Lunt and Matthew 2012) Sufficient oxygen in tissues is needed in an aerobic process If there is little oxygen in tissues hypoxia will occur, which causes a decrease of cells. Hypoxia is a lack of oxygen condition in tissues (Wu and Patricia 2011). Other than decreasing cell energy, hypoxia also causes cell injury which is also caused by free radicals such as Nitrogen Oxide (NO) (Giacco and Michael 2010, Halliwe and Gutteridge 2007)

According to Sherwood (2010), there are four types of hypoxia: hypoxic hypoxia, anaemic hypoxia, circulation hypoxia and histotoxic hypoxia. In a hypoxia condition an animal's body has to keep the oxygenation of tissues. This is conducted through the increase of myoglobin problem with serves as oxygen-storing protein (Fraser et. al. 2006: Puspitaningrum et al. 2010: Sadikin et. al. 2012). According to Wittenberg (2009), myoglobin expression increases in a hypoxia condition and Intensive physical activities. In such conditions myoglobin is very necessary to prevent tissue damage caused by hypoxia.

Myoglobin is an oxygen-storing protein which weighs 16,700 Da and is found in muscle cells. Myoglobin serves to store bound oxygen and increase its transportation to mitochondria, which uses oxygen as cell nutrients (Nelson and

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Cox 2004). Based on previous researches, myoglobin expression occurs not only in muscular tissues but also in non-muscular tissues such as kidney (Frasser et. al. 2006). Myoglobin expression in kidney is found in low-level vertebraes such as goldfish, zebrfish (Cossins et al. 2008) and Brazilian tortoise (Japri 2011). Myoglobin protein is also found in the kidney of green turtles (Dinda 2011).

Kidney is a very important organ in the body which functions to keep the balance of liquid, alkaline acid and salt kidney is sensitive to hypoxia (Sherwood 2008) In serving its function kidney has to keep the oxygenation of tissues going. This can be conducted through a myoglobin increase as an attempt to keep a good oxygenation in the kidney. Therefore, a further research on the effects of hypoxia exposure to myoglobin protein level in animals with higher taxonomy, such as rats (*Rattus norvegicus*) by using spectrophotometry technique and immunodotblot.

**MATERIALS AND METHODS**

This research aimed to gain the level of total protein and myoglobin protein in Wistar rats’ kidneys. This research was conducted at the Animal House and Biochemistry Laboratory in The Faculty of Mathematics and Natural Sciences Universitas Negeri Jakarta. Indonesia, from September 2011 to June 2012.

The observed subjects in this research were 30 male Wistar rats (*Rattus norvegicus*) as available population and 24 were used as sample population. These rats were obtained from The Veterinary Research Board (BALITVET), aged 2.5-3 months and weighed around 100 gr. The parameter used in this research was blood gas test, total protein level and myoglobin protein level in Wistar rats in the treatment and control groups.

The research used Complete Random Design (CRD) which consists of two groups, treatment group (T) and control group (C). Ten per cent of O2 and 90% of N2 gas exposure was applied to the treatment group to the hypoxic chamber for a day (1 P), three days (3 P), seven days (7 P), 14 days (14 P) and 21 days (21 P). The rats in the control group were kept in a normal room. The number of replications in each group in this research was based on the Federer formula calculation. Myoglobin protein detection using spectrophotometry and immunodotblot techniques is explained in the following steps:

a. **Dilution of PBS Buffer Solution 10x, The Making of PBST Solution 20, BSA Solution and Horse Myoglobin Solution**

Dilution of PBS buffer solution 10x was conducted to obtain PBA 1x buffer which was used in this research. To make PBS M buffer solution, the available PBS 10x solution was measured using a measuring cup until it reached 100 ml in volume. Then 900 ml of sterile aquades was added to 100 ml PBS 10x buffer solution. This solution was called PBS 1x buffer solution. The making of 0.05% PBST-20 was conducted by measuring 100 ml PBS 1x buffer solution and adding 0.5 ml of Tween20 solution (Merck) to it. The PBS 1x and PBST-20 solution was also stored in a room temperature (25° C).

The making of horse myoglobin solution was carried out by scaling 0.01 g of horse myoglobin (Sigma 2630-250 MG). After that the myoglobin solution was
dissolved in a measuring pot with 10 ml PBS. This horse myoglobin measured at 100 μg/ml.

b. The Making of Standard BSA and Myoglobin Curves

The making of standard BSA (Sigma) curve was conducted by diluting 1000 μg/ml BSA solution. Dilution was also conducted to BSA solution measuring at 0 μg/ml, 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml, 350 μg/ml, 400 μg/ml, 450 μg/ml, 500 μg/ml. Dilution of BSA solution at different levels aimed to acquire a linear curve rate. Then the absorbance rate of diluted BSA was measured as 280nm using a spectrophotometer (Shimadzu). The result of said measurement was later turned into a standard protein curve and counted to get a total protein level formula.

c. Isolating Protein from Rat Kidneys

Wistar rats kidneys, weighting 250 mg, in this research were scaled using an analytical balance. Then the kidneys were put in a 1.5 ml micro tubes and washed with PBS 1x pH 7 a few times until they looked clear. This was to rid the kidneys from blood. After being washed, 250 μl of PBS 1x pH 7 solution was added to the kidneys. As well as 0.25 μl of 0.01% protease inhibitor which functioned to pulverize tissues to help obtain the protein in it. Next, kidneys were crushed using a pestle pellet. Then centrifuged for ten minutes at 40°C with the speed of 12000 rpm. The supernatant that was formed from centrifugation was moved to an empty and sterile 1.5 ml micro tube. Pellet and supernatant were separated and then stored in a refrigerator at -20°C.

d. Detection Protein in Rat Kidneys using a Spectrophotometry Technique

Spectrophotometry technique was conducted by taking 1 μl of samples which was then diluted in a cuvette which was tilled with 999 μl of sterile distilled water. Its absorbance rate was measured using a spectrophotometer at 280 nm to find out normal protein level and 503 nm to discover the level of myoglobin protein (Bowen 1948). Absorbancy rate was measured twice in two cuvettes. The obtained absorbancy rate was calculated above its average and put in a BSA and myoglobin standard curve formula.

e. Detection of Myoglobin Protein in Rat Kidneys using an Immunodotblot Technique

The flow of myoglobin protein detection using the immunodotblot technique.

f. Gas Blood Test

Hypoxic status of animals experiment can be discovered through the examination of the rat gas. Rat arterial blood was taken at least 500cc. Blood immediately examined using blood gas analysis machine in Laboratory Hematology - blood gas analysis of Hygiene Faculty of Medicine. University of Indonesia.
g. **Statistics Test**

The data of myoglobin level was put on a prerequisite test with a normalcy examination (Kolmogorov-Smirnov). Data was distributed normally. It was followed by a one way ANOVA F test at 0.01. F calculation was higher than F table. The calculation was continued with a Least Significant Difference (SLD) test to find out if there was any real difference. The result of the immunodotblot technique was documented using a digital camera and compared to the reds formed in positive control which were horse myoglobin, and analysed descriptively.

**RESULTS**

1. **Blood Gas**

The gas blood measurement in this research aimed to ensure that rats in the treatment group here in hypoxia. This hypoxia condition could be found by comparing the blood gas test result of rats in the treatment group and that of the rats in the control group. The blood gas test consisted of pH, pCO₂, and pO₂ measurement in the blood of rats in both groups. The results of blood gas test on rats of both groups are shown in Figure 1.

![Figure 1. Graphics of blood gas test results consist of (a) pH, (b) pCO₂, and (c) pO₂ on rats in the hypoxic N₂ 90% treatment and control groups.](image-url)
2. Level of total protein and myoglobin protein of rats

The measurement of total protein and myoglobin protein was conducted through a spectrophotometry technique. To acquire a standard rate for total protein there had to be a measurement for total protein standard using Bovine Serum Albumin (BSA) at 280 nm. While to get a standard rate for myoglobin protein a measurement for standard protein using horse myoglobin (Sigma 2630-250 MG) at 530 nm was needed (Bowen, 1948).

Absorbancy rate of BSA and horse myoglobin was calculated to get a formula for the level of total protein and myoglobin protein. From the calculation result it was known that the formula to get the total protein level of samples was \( Y = -0.003 + 0.0005X \), while the formula to get the myoglobin levels of samples was \( Y = 0.012 + 0.0007X \).

![Figure 2. The absorbancy data of BSA and horse myoglobin was then turned into a standard curve](image)

The absorbancy rate obtained from the 280nm measurement was then distributed to \( Y \) rate at a BSA level formula until the total protein level of each sample was revealed. The total protein and myoglobin protein levels of rat kidneys in the treatment and control groups are in Table 1.
Through a normalcy test (Kolmogorov-Smirnov), it was found that the level of myoglobin protein level data distributed normally. Then the data was put through a one way F ANAVA test at 0.01 and it was found that F calculation was higher than F table (86.42 > 4.25) so the first hypothesis was proven with p < 0.01 ANAVA one way. This shows that there were effects of hypoxia exposure on the myoglobin protein level of rat kidneys. The statistics test was followed by an LSD test to find out any real difference.

3. Level of Myoglobin Protein in Rat Kidneys

Immunodotblot technique was conducted to detect myoglobin protein in the tissues of rat kidneys using nitrocellulose membranes and antibody (Santa Cruz - FL154). The immunodotblot result is more sensitive compared to that of the spectrophotometry technique because immunodotblot used the specificity of reaction between antigen and antibody. Immunodotblot can be seen in Figure 3.

Figure 3 shows that myoglobin protein positively existed in the kidneys of rats in the treatment and control groups as well as positive and negative controls of horse myoglobin (Sigma 2630-250).

Figure 3 shows that myoglobin protein positively existed in the kidneys of rats in the treatment and control groups as well as the positive control while the negative control rats did not show any myoglobin protein. The myoglobin protein showed through a change of color in nitrocellulose membrane, from white to bright red. This showed that there was an attachment between...
myoglobin antigen and myoglobin antibody when being colored using enzymatic dye Amino Etil Carbazole (Sigma).

DISCUSSION

1. Hypoxic status of rat in chronic hypoxic exposure

The physiological condition of rats in the treatment and control groups was found through the result of a blood gas test which consisted of the measurement of pH, pCO\(_2\) and pO\(_2\) in the blood. This result showed that highest pH in the blood of treatment group rats was 7.061 which took place on the third day of hypoxia exposure, while the lowest pH was 6.781 on the 21st day. The pH rate of control group rats was 7.128. The measurement of blood pH showed that the pH of treatment group rats was lower compared to that of the control group rats. This showed that the treatment group rats experienced a hypoxic condition. The blood pH rate in the treatment group was stable throughout 21 days of hypoxia exposure.

In a normal physiological condition, the pH blood rate is a very important physiological parameter because almost all enzyme activities in the body metabolism are influenced by pH late (Cheviron et al. 2012). The pH rate influences biochemical reactions in each organelles of cells so any change in pH can significantly affect the metabolism process in cells (Vinnakota and Kushmerick 2011). This means that an increasing pH rate in the blood of organisms show a hypoxic physiological conditions (Sherwood 2008; Puspitaningrum et al. 2011). In a hypoxic condition an increase of CO\(_2\) occurs. This metabolic CO\(_2\) increase causes an increase of pCO\(_2\) in extracell liquid. According to Sherwood (2010), the transportation of CO\(_2\) in blood can occur in three ways: 10% physically dissolved, 30% of bound hemoglobin which formed hemoglobin carbamino (HbCO2) and 60% in the form of bicarbonate (HCO\(_3\)) through the following chemical reaction. which takes place in red blood cells:

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- 
\]

Through said reaction we can find out that if the level of CO\(_2\) increases, it can be predicted that the level of H\(_2\)CO\(_3\) and H\(^+\) will increase as well, so it can decrease blood pH (Sabatini and Neil 2009). This is the base of theories that in a hypoxic condition blood pH turns into acid. Other than blood pH rate, the rate of pCO\(_2\) in blood can also be a sign that an animal is in a hypoxic condition. The result of a blood gas test showed that the highest pCO\(_2\) rate in blood of treatment group rats was 95.73 mmHg on the 14th day of hypoxia exposure, while the lowest was 69.08 mmHg on the 7th day. Meanwhile, pCO\(_2\) in the blood of control group rats was 65 mmHg. From a blood gas test it was found that the pCO\(_2\) rate in the treatment group rats was higher than that of the control group rats. The high rate of pCO\(_2\) in treatment group rats showed that the rats were in hypoxia.
Tissues or cells continuously conduct metabolism to survive. Generally, in a normal condition metabolism goes aerobically. For this, sufficient O$_2$ is needed and at the end of each metabolism CO$_2$ is generated. In a hypoxia condition with little O$_2$, tissues or cells still have to conduct metabolism. Said metabolism can go anaerobically because of the small number of O$_2$. Anaerobic respiration also generates CO$_2$ (Halliwell and Guttridge 2007). The increase of CO$_2$ generated by metabolism will increase pCO$_2$ in blood (Sood et al, 2010).

In Figure 1 Graphic (b) we can see that the pCO$_2$ in rat blood was stable during a hypoxia exposure for one to three days, but on the seventh day of exposure the pCO$_2$ rate of blood decreased to 69.08 mmHg, close to the pCO$_2$ of normal rats (control group). This showed that on the seventh day of exposure, rats started to adapt to hypoxia, but on the 14th and 21st day pCO$_2$ rate increased again and it showed that metabolism-generated CO$_2$ was on the rise.

Other than pH and pCO$_2$, another physiological trait which shows that rats are in hypoxia is pO$_2$ rate (Sherwood 2008). A gas blood test showed that the highest pO$_2$ rate in treatment group rats was 27.98 mmHg, which took place on the 21st day of hypoxia exposure, while the lowest was 14.98 mmHg which occurred the third day.

The pO$_2$ rate of control group rats was 25 mmHg. On the first day of hypoxia exposure, the pO$_2$, blood rate of treatment group rats was still above that of normal rats (of the control group). However, this condition did not show that rats were in hypoxia because the rates of pH and pCO$_2$ in blood did not show hypoxia. On the third day of hypoxia exposure, the pO$_2$ rate in the blood of treatment group rats decreased below that of normal rats (control group) but it increased again on the seventh day; then it stayed stable until the 21st day. The low pO$_2$ rate in rat blood on the third day was caused by the mere 10% of O$_2$ given to rats during hypoxia treatment. The low rate of O$_2$ also caused the low rate of PO$_2$.

Through a blood gas test we found out that all sample rats in the treatment group suffered from hypoxia. On the seventh day of hypoxia exposure said rats started to adapt to hypoxia, which was shown by their rates of pH, pCO$_2$, and pO$_2$ that were close to those of normal rats (control group). It showed that tissues tended to adapt to low O$_2$ environments starting the seventh day of hypoxia exposure.

2. **Myoglobin protein levels was increased in chronic hypoxic exposure rat Kidneys**

From Table 1 we can find out that the level of myoglobin protein is smaller than that of total protein. This shows that myoglobin protein exists in a small number in tissues. However, myoglobin protein has a significant role in said tissues. Also from Table 1 it is known that the total protein level in the control group was higher than that of the treatment group. This was caused by the undisturbed metabolism of control group rats which generated more total protein (336.75 pg/ml). On the contrary, the metabolism of treatment group rats
experienced a disturbance caused by the low consumption of O$_2$, which in turn caused a smaller number of total protein.

Still on Table 1, it shows that the myoglobin protein level in the treatment group was higher than that of the control group on the 14th (28.57 pg/ml) and 21st day (33.40 pg/ml). This shows that in a hypoxia condition with little O$_2$, the amount of myoglobin increased in relation to its function as oxygen storing and binding protein.

3. Myoglobin protein in chronic hypoxic exposure rat kidneys

Immunodotblot is a technique which can also be used to detect myoglobin protein. However, this technique is more sensitive than spectrophotometry because it uses an antigen-antibody reaction. What indicated the existence of myoglobin protein in the immunodotblot technique was the red in nitrocellulose membrane which was previously white. The red color as a positive indicator in an immunodotblot test was caused by the chain reaction between antigen-antibody and secondary antibody that was H$_2$O$_2$.

The result of immunodotblot test that is shown in Figure 2, it is known that all samples, from both the control group and treatment group, show a positive result marked by a red color in nitrocellulose membrane. In nitrocellulose membrane, the positive control used horse myoglobin (Sigma 2630-250 MG), whose generated color was thicker than all other colors. This was because the horse myoglobin used came from muscular tissues which were indeed loaded with a lot of myoglobin. The negative control, on the contrary, did not generate a positive result. This was because no antigen was used in the negative control.

In Figure 2 we can see that the color in the treatment group is thicker than that of the control group, especially on the 14th and 21st day. This shows that more myoglobin protein was generated in the kidneys of rats in the treatment group than in the control group. This was in line with the spectrophotometry result.

4. Why Myoglobin protein is in chronic hypoxic exposure rat kidneys?

A rat's kidney is an important organ which functions to keep the balance of liquid, amino acid and salt in the body (Sherwood 2008). To serve this function, kidney needs enough energy in the form of ATP. The process of ATP making occurs in mitochondria and needs sufficient oxygen.

In this research, myoglobin protein was detected in rat kidneys through both spectrophotometry technique (Table 1) and immunodotblot technique (Fig. 3). However, there was more myoglobin protein in the treatment group than in the control group. This was because myoglobin protein functioned to bind and store oxygen which would be produced more to counter a lack of oxygen condition.

There was a probability that myoglobin protein in kidney was in the smooth muscular tissues and epithel tissues which formed the kidney. According to Qiu et.al. (1998), myoglobin protein is not only spread in skeletal muscles and
heart muscles but also in smooth muscles. In kidney there are layers that form an afferent arteriole wall which consists of tunica media which itself consists of smooth muscle layers. Therefore, it is known that myoglobin protein in kidney is most likely in the tunica media that forms afferent arteriole. However, according to Fraser et. al. (2006). The amount of myoglobin in smooth muscle tissues is less than that in the heart muscle and skeletal muscle.

Myoglobin protein in the kidney tissues is one physiologically adaptive form of the organ. This protein helps with oxygen supply transportation to mitochondria in facilitating oxygen diffusion, in accordance with its function as intermediary protein (Fraser et al. 2006; Wittenberg 2009). Inside mitochondria, oxygen supply is used as an ATP forming source to serve an array of kidney functions. This research the following conclusions have been drawn: Myoglobin protein exists in rat kidneys. In both treatment and control groups, which was detected through spectrophotometry and immunodotblot techniques and hypoxia exposure affected myoglobin protein concentration in rats’ Kidneys.

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