

The effect of intermittent hypobaric-hypoxia treatments on renal glutathione peroxidase activity of rats

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Abstract. Many people living at high altitudes experiencing a condition called intermittent hypobaric hypoxia (IHH). Some people even create IHH condition as an exercise for pilots, athletes, and mountaineers. In this experiment, we aimed to determine whether the protective effect of IHH is mediated through glutathione peroxidase (GPX) enzyme. The experiment's sample is two-month-old healthy Sprague-Dawley rat kidneys weighing 200–250 g. Intermittent hypobaric hypoxia treatment is done using a Hypobaric Chamber type I that can mimic air pressure at certain altitudes: 35,000 (one minute), 30,000 (three minutes), 25,000 (five minutes), and 18,000 (30 minutes) feet. The rats were divided into five treatment groups, including a control group, hypobaric-hypoxia group, and intermittent hypobaric-hypoxia 1x, 2x, and 3x groups with each group consisting of three rats. The specific activity of GPX was measured using RANDOX and RANSEL methods. The statistical analysis of one way-ANOVA did not show significant differences between the groups ($p > 0.05$), although specific activities of the renal GPX of rats exposed to hypobaric-hypoxia were higher than the control group. This may be caused by the other antioxidants' activities. In conclusion, the IHH treatment did not affect GPX activity in the rat kidneys.

1. Introduction

A short-term oxygen supply delay may induce changes in our body, including an oxidative stress condition through the formation of reactive oxygen species (ROS). This is quite common in organisms with aerobic metabolism to generate ROS, causing oxidative stress. Both excessive or a lack of oxygen can generate ROS. This situation is dangerous because all the ROS can affect the normal metabolism in the body [1]. ROS can cause cell membrane damage through lipid peroxidation and protein oxidation in carbonyl substances, and thus may cause DNA damage and disrupt ATP production [1,2]. The level of antioxidants may likewise decrease as a result [1]. However, not every hypoxia condition will lead to cellular damage.

Intermittent hypobaric hypoxia (IHH) is a condition in which the body is exposed to a repetitive hypoxic or low oxygen-level condition due to a reduced barometric pressure for a certain period of time [1]. A chronic condition is usually experienced by people who live at a high altitude, such as in mountains or areas 1,500 meters above sea level (asl) [1,2]. This exposure to hypoxic conditions is interspersed with some normoxic period between each episode. The training of IHH is suggested by the sports medicine community and has long been used to enhance the performances of pilots, mountaineers, and athletes [3,4]. Some outcomes have suggested that IHH has health benefits by increasing the body's tolerance against hypoxic conditions. Therefore, many studies have been done to



prove this hypothesis and to find the mechanism behind it. Since then, many findings have shown evidence that IHH pretreatment could yield protective effects for some organs.

In the first experiment done by Shi *et al.* [5], chronic IHH treatment was able to alleviate and prevent collagen-induced arthritis (CIA) in rats. There were five groups: a pre-treatment group, a pre-control group, a post-treatment group, a post-control group, and a blank control group [5]. The pre-treatment group showed a lower incidence rate of CIA than the pre-control group ($p < 0.05$) [5]. The pre- and post-treatment rats also showed alleviated ankle joints with higher apoptotic rates of synovial cells and increased T lymphocytes rather than the control ones ($p < 0.05$) [5]. It is thought that the protective effect occurred through the apoptotic mechanism [5]. In another experiment by Shi M *et al.* [6], the incidence rate of CIA in rats pretreated with IHH was lower than in the non-exposed rats ($p < 0.05$). This is hypothetically thought due to other findings that showed reductions in serum and synovial tissue TNF- α , IFN- γ , NF- κ B, IL-4, and IL-17, and peripheral blood CD4/CD8 T-lymphocytes in rats exposed to chronic IHH, different from the non-exposed ones ($p < 0.05$) [6]. This particular finding may support the theory that the protective effect of chronic IHH is mainly on the inflammatory reaction [6]. It is thought that chronic IHH treatment may induce low HIF-1 α activity [6].

A study by Guan Y *et al.* [7] showed that chronic IHH has a protective effect toward the heart against ischemia and reperfusion complications, i.e., arrhythmia and infarction. Sympathetic stimulation through β 1-receptors may have a positive inotropic effect, but it may also enhance infarction and arrhythmia during acute ischemia [7]. The cardioprotective mechanism suggested is due to the attenuated receptor activity of the beta-adrenergic receptor [7]. To assess the activity, the action potential duration and contractility were measured, and the results showed a longer duration but no contractility difference than the control group [7]. It was thought to be related to the down-regulation of the receptor [7].

Zhu XH *et al.* [3], found that intermittent hypoxia (IH) promotes neurogenesis in the adult hippocampus, which has an anti-depressant effect. The effect was measured in three animal models screening for anti-depressant activity: the chronic mild stress (CMS) test, the forced swimming test (FST), and the novelty suppressed feeding (NSF) test. All three showed a reversal in the depressive characteristics [3]. Although many studies have demonstrated IHH's benefits for certain organs, many researchers are still curious as to whether these protective effects come from the actions of several antioxidants. Since hypoxic conditions may trigger the expression of erythropoietin (EPO) as a compensation mechanism against a lack of oxygen and EPO has antioxidant effects, it remains controversial whether antioxidants likewise contribute to help the adaptation of those organs. In a study by Costa DC *et al.* [4], IHH pre-treatment reduced the oxidative stress and apoptotic activity post-administration of kainate, a model of brain oxidative stress, mimicking the outcome of hypoxia. The reduced apoptosis can be measured by looking at the activity level of apoptotic proteins like caspase-3 and NF- κ B that were decreased [4]. Thus, IHH is thought to enhance the brain antioxidant capacity and promotes neuroprotection in kainate-induced oxidative injury [4].

Evidence on the protective effect of IHH through many mechanisms has grown and developed recently, but there are still very limited data regarding its relationship with antioxidant enzyme glutathione peroxidase (GPX), one of the most efficient antioxidant enzymes in the body [8]. In the kidney, GPX works extracellularly [8]. The kidney itself is an organ affected by systemic conditions. It receives 20% blood from the heart, but the pO_2 can be as low as 5 mmHg in the medulla, which is different from in the cortex (50 mmHg) [9]. The oxygen is used to carry out complex functions; hence, low-oxygen areas, such as in the medulla, could be rather susceptible toward hypoxia [9]. In this experiment, the writer aims to determine whether the protective effect of IHH is related to the antioxidant activation, specifically GPX enzyme, through measuring its activity in the kidney. By conducting this experiment, it can be determined whether the protective effect of IHH is mediated by GPX activity. To identify the effect of IHH toward the kidneys' GPX activity, there are two more specific objectives: measuring the protein concentration of rats' kidney tissues after exposure to 1, 2, 3, and 4 times of hypobaric hypoxia and control, and measuring and comparing GPX-specific activity

in the kidney tissue of rats after the same frequency of exposure of hypobaric hypoxia and control groups.

2. Materials and Methods

The study is a continued process from the previous experiment done by a prior researcher. It employed an experimental design using primary sources and was held in August 2015 at the Biochemistry Laboratory of the Biochemistry Department, Faculty of Medicine Universitas Indonesia (FMUI). The samples were stored kidney organs from the previous experiment, which were kept in Biochemistry Department FMUI. The animal criteria are two-month-old healthy male Sprague-Dawley rats, each with a weight of 200–250 grams. To count the minimum number or sample size of the rats for each group, the writer used Mead's formula, which is commonly used in animal study, in which the total information in the experiment uses N experimental units. In general experimental situations, there are three components that comprise the total variation, which are the treatment component or group (T) that is related to the asked questions, the blocking component (B) that depicts environmental effects allowed for in the design, and the degrees of freedom (df) of the error component (E) that have scores ranging between 10 to 20. The components including N , B , and T are based on the df ; hence, each component has to be added with minus 1. The formula is as follows: $E = N - B - T$. In this experiment, no environmental effects are allowed, which means the B is equal to 0 (zero). Thus, the equation was as follows: $E = N - T$.

The N was the total of samples used, consisting of the total number of group times the number of samples for each group. In this case, the sample was represented by X since it is the variable that needed to be counted. This resulted in $N = 5X - 1$ (explained above). The score for E is chosen for 10 and because the total treatment groups are 5, the T was 4 (explained above). So, when the numbers were inserted, the equation became: $10 = 5X - 1 - 4$; $15 = 5X$; $X = 3$. Therefore, the total number that should be used as the sample size in each group is equal to or more than 3. Hence, the total number organs from treated rats were 15. Using Mead's formula, the total number in each group was equal to or more than 3.

There were five groups: the control group was not exposed to any hypoxia condition, the hypobaric-hypoxia group was exposed to a hypoxia condition only on the first day, the intermittent hypoxia 1x group was exposed on the first and eighth days, the intermittent hypoxia 2x group was exposed on the first, eighth, and fifteenth days, and the intermittent hypoxia 3x group was exposed on the first, eighth, fifteenth, and twenty-second days. The hypoxia condition was created using a hypobaric chamber mimicking air pressure at 35,000, 30,000, 25,000, and 18,000 feet and each height was undergone for 1, 3, 5, and 30 minutes, respectively, for the type-I Chamber flight profile. This was done by the previous researcher. After the rats were euthanized and the kidneys were taken, they were stored in a freezer at a temperature of -86°C . A chunk of the tissue that weighed 100 mg was then added to 500 μl of phosphate buffer saline (PBS) solution at a pH of 7.2. After being homogenized, the mixture was added again to 500 μl of PBS and then centrifuged 3500 RPM for 10 minutes. The supernatant was then used to measure the protein using Warburg-Christian's method. There are seven groups comprising of one blank and six BSA tubes with their standard concentrations: 50, 100, 200, 300, 400, 500 ($\mu\text{g/ml}$). The blank tube had 1 ml aquadest added to it, while the six BSA standard tubes were added to 0.950, 0.900, 0.800, 0.700, 0.600, and 0.500 ml of aquadest, respectively. Next, the standard tubes were added with 1 mg/ml concentration of standard BSA stock with volume of 0.050; 0.100; 0.200; 0.300; 0.400; 0.500 ml, subsequently. The absorbance was measured in the wavelength of 280 nm.

To measure the GPX activity of the rats kidney tissue, the RANDOX and RANSEL methods were used. Each sample must be made in duplo. First, each sample was taken for 10 μl . It had to be diluted 50 times with NaCl solution; hence, the volume of NaCl required was 490 μl to reach the total volume of 500 μl . The mixture was then homogenized by being put into the vortex until it was homogenous; no specific timing was required. From the total 500 μl , 20 μl of the homogenized mixture was taken and was moved to a cuvette. Because each sample was made in duplo, the total volume used for two

cuvettes was 40 μ l. Then, the first reagent (R1) should be prepared first. There were two ingredients: R1A and R1B, which functioned as the reagent and the buffer to dilute the reagent, respectively. There were eight bottles of R1A, each with a volume of 10 ml. The reagent was stable for 48 hours at 2–8 °C or 8 hours at 12–25 °C. The R1B was stable until the expiry date and required storage at 2–8 °C. There was one bottle of R1B with a volume of 70 ml. To dilute the reagent, the procedure was only to put 10 ml of R1B into each bottle of R1A. Then the R1 was ready. The second reagent (R2) consisting of cumene hydroperoxide also required dilution. The preparation was to add 10 ml of saline to 10 μ l of R2. After adding it, the mixture should be shaken vigorously since cumene is hardly dissolved. The R2 was stable when stored at 2–8 °C. The R3 is a diluting agent to dissolve blood, but in this experiment there was no blood used; hence, the reagent was not used. After preparing the required reagent (R1 and R2), the procedure can be continued. The R1 should be added (as much as 1 ml) to the 20 μ l diluted sample. Then, 40 μ l R2 was added for each cuvette. Following the administration of R2, the stopwatch was started immediately and while waiting for 1 minute, the mixture was mixed using a pipette in an up and down method three times. Each cuvette was measured in absorbance twice; one minute after adding R2 and two minutes after the first absorbance reading. The absorbance measurement was using a spectrometer in the wavelength of 340 nm.

The absorbances of protein and GPX measurements were processed using Excel. For the BSA standard curve, the average absorbance from the two values was counted, and then the linear regression was made. Using Excel, the linear regression of the average data can be illustrated in a line graph, and the A and B can be calculated by using the slope and intercept, respectively, with the X and Y axis resembling the standard concentration of BSA and the average absorbance subsequently. The slope is used basically to count the gradient of the line, whilst the intercept is used to find the value of the dependent variable when the independent variable is zero. The linear equation is: $Y = AX + B$. There are two major steps to calculate the protein concentration: making the standard curve BSA and counting the protein concentration. The first step was done with the results shown above. The R or correlation value of this graph was calculated using Excel (CORREL). If the value approaches 1 and is positive, it means that the average absorbance and the standard concentration have similar propensities or are nearly identical to one another. Increased concentrations may result in increased absorbance and vice versa. Next, the protein of each sample in the experiment was also calculated by measuring the absorbance of each sample's protein twice. The average of each sample's absorbances was the "y" in the previous equation. The "x," which represents the protein concentration (μ g/ml), can then be calculated for each sample. After converting the unit to mg/ml, each sample's protein concentration had to be multiplied by 100 (due to the frequency of dilution) to obtain the total protein concentration. To measure the GPX activity, we first needed the absorbance measurement data. There were five sample categories to be processed. The delta absorbance per minute of both average absorbance values (since each sample is made in diplo) should be counted by dividing the average value's differences by 2 because from the first to the second absorbance, there was a time gap of two minutes. For the GPX activity, the Δ absorbance/minute can be counted using the following formula: (Average values of absorbance 1 minute – Average values of Absorbance 2 minute)/2. Then, to count the enzyme activity, the formula is as follows: (Δ Absorbance of samples – Δ Absorbance of blank) x 8412 x 50.

The specific enzyme activity is found by dividing the enzyme activity with the total concentration. The units had to be converted and adjusted first before each calculation. For data interpretation, if the specific activity of GPX was normal using the Shapiro-Wilke's test and homogeneity using Levene's test, the parametric test using analysis of variance (ANOVA) was done with the subsequent least significant difference (LSD) test as the post-hoc test. If the data were not normal or homogenous, a non-parametric test, such as the Kruskal-Wallis test was done and the post-hoc test was the Mann-Whitney test.

3. Results and Discussion

3.1 Results

The measurement of the protein resulted in a linear graph with equations of $Y = 0.0005X + 0.0007$ and $R^2 = 0.99976$. The X-axis represented the standard BSA concentrations, and the Y-axis represented the absorbance measurements of standard protein at 280 nm (Figure 1).

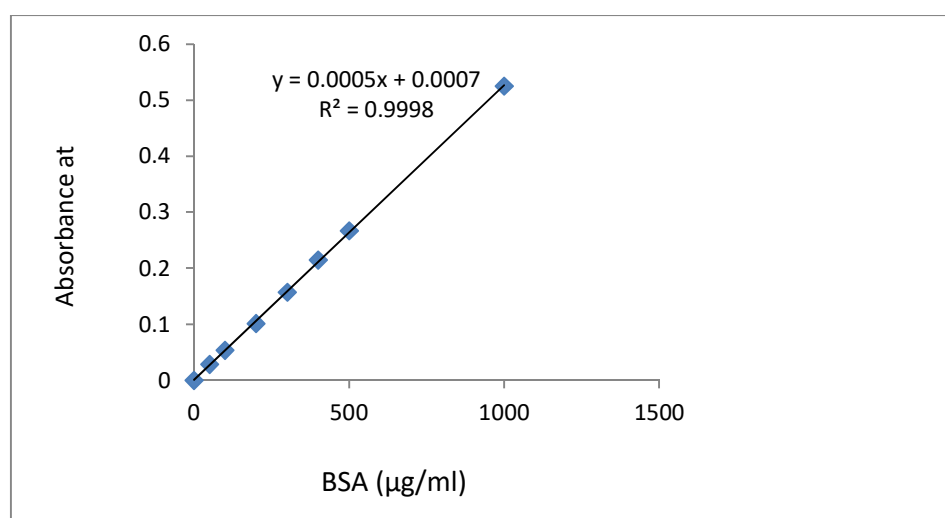


Figure 1. Standard Curve of Bovine Serum Albumin (BSA) using concentrations of 50, 100, 200, 300, 400, and 500 (µg/ml)

The specific activity is shown in the Table 1 below.

Table 1. Specific activity of glutathione peroxidase in Kidneys of rats

Samples	Mean GPX Specific Activity (U/mg)	Standard Deviation
Control	0.068	± 0.044
Hypobaric-Hypoxia	0.158	± 0.070
Intermittent Hypobaric-Hypoxia 1x	0.158	± 0.077
Intermittent Hypobaric-Hypoxia 2x	0.091	± 0.072
Intermittent Hypobaric-Hypoxia 3x	0.137	± 0.074

After gaining each sample's specific activity, the data were then managed in SPSS software. From the normality test using the Shapiro-Wilke's test, the p-value was 0.717, which means that it was >0.05 . Hence, all the individual-specific activity data were in the normal range to undergo parametric tests. Next, using Levene's test, the homogeneity of variances of the data had a p-value of 0.856, likewise above 0.05. Therefore, we can conclude that the individual-specific activity data were normal and homogenous. Then, the data were measured in a parametric way using a one-way analysis of variance (ANOVA) test. The p-value was 0.422, >0.05 . In conclusion, there was no variation amongst the group means of the individual-specific activity data of GPX; thus, the post-hoc and correlation tests were not required (Figure 2).

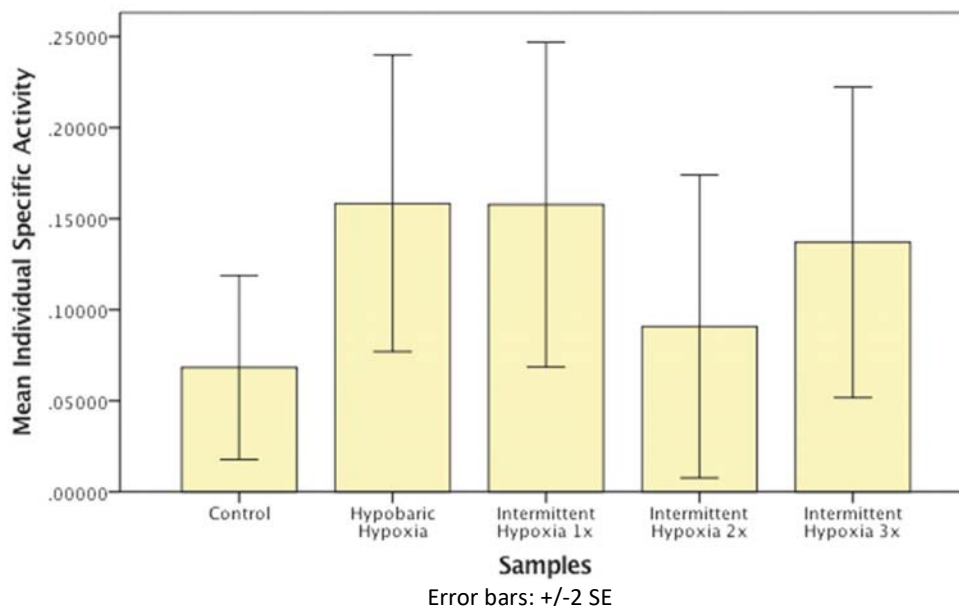


Figure 2. Bar graph with the standard error of mean specific activity of glutathione peroxidase in the kidney. Control group, hypobaric hypoxia: 1x treatment in a type 1 hypobaric chamber, intermittent hypoxia 1x: 2x treatment, intermittent hypoxia 2x: 3x treatment, intermittent hypoxia 3x: 4x treatment.

3.2 Discussion

In this experiment, we aimed to identify whether the IHH treatment could provide protection that could lead to an increase in an organ's tolerance toward hypoxia conditions through the enzyme antioxidant GPX. First, from the line graph and the histogram of the group-specific activities of GPX, we can see that the control group had the lowest GPX-specific activity compared to the other groups with IHH treatments. Although there was no gradual increment of the GPX-specific activity from the hypoxic control group to the 4x hypoxic group and instead a steep fall from the 2x hypoxic group to the 3x hypoxic group, there was finally a sudden surge in the fifth group. From these data, it seems that there are still some effects of hypobaric-hypoxia treatment toward renal GPX activity since the hypoxic groups have higher results than the control group. Nevertheless, using SPSS, we can say that the initial hypothesis that there would be a positive relationship between IHH and GPX is rejected. The ANOVA test shows that the IHH does not affect the number of GPX-specific activities. Thus, the graph of the GPX-specific activity does not represent the outcome according to the hypobaric-hypoxic treatments.

There are many possible factors explaining why the hypothesis was rejected. First, the minimal sample numbers due to feasibility reasons may play a role. For each category, there were only three samples. Although, according to Mead's formula, the number is sufficient, the data would be better distributed in a larger number of samples. The imbalance in volume for each cuvette due to mishandling the pipette may affect the fluctuation of sample values even in one sample category. The other factor is perhaps the difference in time exposure during the procedure. In many other experiments, the treatment is done every day, whilst in this experiment the treatment was done once per seven days. Other possible factors may also ensue.

There are abundant antioxidants in the human body comprised of enzymatic and the non-enzymatic ones. Examples of enzymatic antioxidants include GPX, SOD, and CAT [10,11]. Other than these major enzymes, thioredoxins, peroxiredoxins, and glutaredoxins may likewise play a minor role [11]. GPX fights mostly hydrogen and lipid peroxides. Both substances are induced by hypoxia [10,11].

There are other enzymes having a similar capacity, especially to hydrogen peroxide, such as CAT, thioredoxin reductases, thioredoxin peroxidases, and glutaredoxins [11]. In this case, perhaps the effect of GPX has been taken over by the other enzymes; hence, we cannot see its real, specific activity. The level of GPX specific activity is also affected by the availability of the other cofactors of the GSH-GSSG cycle. Espinoza SE *et al.* stated that in a chronic oxidative stress condition, GPX activity may be increased due to the upregulation of this antioxidant enzyme [12]. However, during acute oxidative stress conditions, the enzyme is outnumbered by myriad free radicals, which may cause the depletion of GSH, hence causing GPX to run out [12]. In this experiment, the treatment was not done in a chronic method, but instead was done intermittently; hence, the rats had repeated acute exposure. GSH itself supports numerous cellular processes, such as cell differentiation, proliferation, and apoptosis [12]. GSH levels can be disrupted by many factors. One of them is inherited or acquired problems in any supporting structure within its pathway, such as the enzymes, transporters, signaling molecules, or transcription factors. Exposure to reactive chemicals or metabolic intermediates could also cause the drawbacks of this substance. The lifecycle of GSH from its intracellular synthesis to its extracellular metabolism is called the gamma-glutamyl cycle. Any disruption during this cycle may cause GSH to not function properly. For example, some amino acids are required as the precursor of this substance. A lack of them due to diseases and inadequate nutritional intake may produce less GSH [13]. In addition, the substance NADPH is likewise required as a cofactor to convert GSSG back to GSH [11]. If there is depletion of NADPH, the cycle may also be disrupted and there will be less GSH formed. Hence, GPX may not exert its effect at a high level.

4. Conclusion

Although the results of the group-specific activity indicated that the GPX level in the kidneys was roughly increased along with more frequent hypobaric-hypoxic treatment, IHH treatment does not affect GPX activity in the kidneys of rats.

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