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Effects of Hypoxic Preconditioning on Oxidant-Antioxidant Systems in Rat Lung

Sıçanlarda Hipoksik Ön Koşullamanın Akciğerdeki Oksidan-Antioksidan Sistemler Üzerine Etkisi

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ABSTRACT Objective: Hypoxic preconditioning allows cells to gain resistance to hypoxic damage. There are a limited number of studies suggesting that hypoxic preconditioning increases antioxidant capacity in the lung. In this study, we aimed to evaluate effects of hypoxic preconditioning on oxidant/antioxidant systems in rat lung. **Material and Methods:** Rats were divided into 4 groups: control, preconditioning (PC) (10% O_2), severe hypoxia (SH) (7% O_2) and PC + SH. The parameters related to oxidative stress and antioxidant defense mechanisms, which are malondialdehyde (MDA) levels, total oxidant system (TOS), total antioxidant capacity (TAC), oxidative stress index (OSI), superoxide dismutase (SOD) and glutathione (GSH) activity, were measured. Results were evaluated using the One-Way ANOVA and t-test; $p<0.05$ was considered significant. **Results:** Compared with the control group, MDA levels decreased in all hypoxic groups; decrements in PC and SH groups were statistically significant. Compared with the control group, levels of TOS showed a significant increase in the PC+SH group. OSI of PC+SH group was significantly higher than other groups. There was no significant difference in TAC levels between the groups. Compared to other groups, SOD activity showed a significant decrease in the PC+SH group. GSH levels showed a significant decrease in PC+SH group compared to both control and PC group. **Conclusion:** Our findings suggest that hypoxic preconditioning does not have an effect on antioxidant defense systems in lungs, but severe hypoxia does affect oxidant/antioxidant systems.

Keywords: Hypoxia; hypoxic preconditioning; lung; reactive oxygen species; oxidant/antioxidant systems

ÖZET Amaç: Hipoksik önkoşullama, hücrelerin hipoksik hasara karşı direnç kazanmasını sağlar. Hipoksik önkoşullamanın akciğerdeki antioksidan kapasiteyi arttırdığı yönündeki çalışmalar sınırlı sayıdadır. Bu çalışmada sıçan akciğerinde hipoksik ön koşullamanın oksidan/antioksidan sistemler üzerindeki etkilerinin değerlendirilmesi amaçlanmıştır. **Gereç ve Yöntemler:** Sıçanlar; kontrol (K), ön koşullama (ÖK) (%10 O_2), ağır hipoksi (AH) (%7 O_2) ve ağır hipoksi+ön koşullama (AH+ÖK) olmak üzere 4 gruba ayrılmıştır. Dokularda oksidatif stres ve antioksidan savunma mekanizmaları ile ilişkili parametreler olan malondialdehit (MDA) düzeyi, total oksidan sistem (TOS), total antioksidan kapasite (TAK), oksidatif stres indeksi (OSİ), süperoksit dismutaz (SOD) and glutatyon (GSH) aktiviteleri ölçülmüştür. Sonuçlar Tek Yönlü ANOVA ve t testi kullanılarak değerlendirildi; $p<0,05$ değerleri anlamlı kabul edildi. **Bulgular:** Kontrol grubu ile karşılaştırıldığında, MDA seviyeleri tüm hipoksik gruplarda azalma göstermiştir; ÖK ve AH gruplarındaki bu azalma istatistiksel olarak anlamlıdır. Kontrol grubu ile karşılaştırıldığında, TOS düzeyleri AH+ÖK grubunda belirgin bir artış göstermiştir. AH+ÖK grubunun OSİ değerleri diğer gruplara göre anlamlı düzeyde yükselmiştir. Gruplar arasında TAK düzeyleri bakımından anlamlı bir fark bulunamamıştır. Diğer gruplara kıyasla, SOD etkinliği ağır hipoksi+ön koşullama grubunda önemli bir azalma göstermiştir. Kontrol ve ÖK grubuyla kıyaslandığında GSH düzeyi AH+ÖK grubunda anlamlı bir azalma göstermiştir. **Sonuç:** Bulgularımız hipoksik ön koşullamanın akciğerlerde antioksidan savunma sistemlerin üzerinde etkili olmadığını ancak ağır hipoksinin oksidan/antioksidan sistemleri etkilediğini göstermektedir.

Anahtar Kelimeler: Hipoksi; hipoksik ön koşullama; akciğer; reaktif oksijen türleri; oksidan/antioksidan sistemler

An organism can be faced to hypoxic conditions either due to natural reasons such as living in an high altitude or travelling to such places, or due to pathological reasons such as ischemia, cardiac arrest or loosing blood. Hypoxia/ischemia triggers various processes that cause changes in cellular membrane functions, metabolism, intracellular signaling and even cell morphology.[1,2] Hypoxia causes a decrease in ATP levels by blocking phosphorylation/oxidation of glucose. Moreover, oxidative damage may occur under hypoxia due to increased production of reactive oxygen species (ROS) as well as decreased activity of antioxidant defense systems. Under normal physiological conditions, ROS that are formed by metabolism are highly inactivated via both cellular and extracellular antioxidant defense systems.[3-5] Oxygen is normally reduced to H_2O by cytochrome oxidase. However, during hypoxia low oxygen levels cause an accumulation of reducing equivalents in the mitochondrial respiratory chain. This reductive stress leads to autoxidation of one or more mitochondrial complexes, thereby increasing the formation of ROS, resulting in oxidative stress.[6-9] Therefore, hypoxia related oxidative stress damages cellular defense systems such as antioxidants, and decreases their activation.[5,10]

Extensive hypoxia may cause organism to develop protective mechanisms. In fact, acclimatization, which is the tolerance to hypoxia in individuals who live in high altitudes for a long time, is a typical example to this condition. Acclimatized individuals show several adaptive changes at molecular, biochemical and cellular levels. For example, during the acclimatization processes, there is an increase in excitation potential of peripheral chemoreceptors and in capacity of respiratory membrane diffusion.[11] This situation leads to an increase in pulmonary ventilation. Erythropoietin (EPO) production, which is stimulated due to hypoxia, increases the production of erythrocytes.[12] 2,3-diphosphoglycerate, which is produced under anaerobic glycolysis, decreases the affinity of hemoglobin for O_2. By these adaptive responses, oxygen transportation to peripheral tissues increases and oxygen consumption of the tissues is regulated. Upregulation of mitochondrial enzymes also increases the oxygen consumption.[12]

Ischemic and/or hypoxic preconditioning is the process in which the cells are exposed to hypoxia or ischemia for a short time. This process causes the cells to acquire resistance to lethal levels of hypoxic/ischemic damage. In recent years, several in vitro and in vivo studies supplied evidence that hypoxic or ischemic preconditioning provides protective effects in various tissues including kidney, lung, heart, and brain.[13-17] Moreover, protein kinase C, Src protein tyrosine kinase and Janus kinase signaling pathways and transcription factors such as nuclear factor-kappa B (NF-kB)[21] and signal transducer and activator of transcription (STATs) have been shown to play a role in the late phase of hypoxic/ischemic preconditioning in heart muscle, and also, cyclooxygenase 2 and inducible and neuronal nitric oxide synthases were shown to increase in this late phase of preconditioning.[9,18-24]

Lung is amongst the first affected organs by hypoxic conditions because of its direct contact with oxygen. Severe hypoxia has been shown to cause pulmonary edema in several studies.[25,26] Increases in pulmonary vasoconstriction and vascular permeability, and decreases in alveolar clearance as a result of structural and functional impairments of alveolar-capillary membrane are among the underlying mechanisms of hypoxic pulmonary edema.[27-29] Reduction of oxygen supply to peripheral tissues due to hypoxic pulmonary edema can lead to multiple organ failure. Therefore, the lung is the most important rate-limiting organ for the survival of the organism in the presence severe hypoxia.

In recent years, Zhang et al. have shown that whole body hypoxic preconditioning prolongs the survival time in severe hypoxia.[15,30] These researchers have suggested that the hypoxic preconditioning model they used decreases the increased vascular permeability and pulmonary edema by protecting the respiratory membrane cells, especially type I alveolar epithelial cells which are more susceptible to hypoxia. Shukla et al. also have

shown an increase in antioxidant capacity in lungs with a hypoxic preconditioning model by using cobalt and have suggested that this process may protect cells against oxidative damage induced by hypobaric hypoxia.[4]

However, the effects of hypoxic preconditioning on oxidant/antioxidant systems in lungs have not yet been fully clarified. In this study, possible effects of hypoxic preconditioning on lung's antioxidant/oxidant systems were investigated.

MATERIAL AND METHODS

Animal experiments were carried out according to the guidelines issued by Gazi University Animal Experiments Local Ethics Committee (G.U.E.T-12.082). Present study was conducted in accordance with the ethical standards of Declaration of Helsinki, and animal rights were considered. In this study, 25 adult male Wistar albino rats (supplied by Experimental Animal Center of Gazi University) weighting between 200-220 g were used. Rats were randomly divided into 4 groups, as follows: control (C), preconditioning (PC), severe hypoxia (SH), preconditioning+severe hypoxia group (PC+SH). The control group was exposed to normal atmospheric conditions for 3 days, and lung tissue samples were taken under anesthesia on day 4. The preconditioning group was placed in to a hypoxic chamber where they were exposed to 10% O_2 (~5730 m) 2 hours per day for 3 days, and lung tissue samples were taken under anesthesia on day 4. The severe hypoxia group was exposed to normal atmospheric conditions for 3 days, and on day 4, rats were placed in to a hypoxic chamber where they were exposed to 7% O_2 (~8410 m) 3 hours, and lung tissue samples were taken under anesthesia. The preconditioning + severe hypoxia group, on the other hand, was placed in to a hypoxic chamber where they were exposed to 10% O_2 2 hours per day for 3 days, and on day 4, subjects of this group were placed in to a hypoxic chamber where they were exposed to 7% O_2 3 hours, and lung tissue samples were taken under anesthesia afterwards. All groups were anesthetized with a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg).

DETERMINATION OF MALONDIALDEHYDE (MDA) AND TOTAL GLUTATHIONE (GSH)

Samples were weighed and homogenized in ice-cold trichloroacetic acid (9 ml 10% trichloroacetic acid per 1 g tissue).[31] Following the homogenization, samples were centrifuged at 4,000 rpm and 20°C for 15 minutes. Supernatants were collected and centrifuged at 4,000 rpm and 20°C for 8 minutes; then supernatants were collected and aliquoted for MDA and GSH assays.

Determination of MDA

MDA is an indicator of free radical production and lipid peroxidation. MDA levels were measured by thiobarbituric acid reactive substances (TBARS) assay.[31] Briefly, 10 µl of 1% butylated hydroxytoluene and 750 µl of 0.67% thiobarbituric acid were added to 750 µl of supernatant and then vortexed. The mixture was incubated in a 120°C water bath for 15 minutes for color development and then centrifuged at 3000 G for 8 minutes. The absorbance values of the samples was measured spectrophotometrically at 535 nm. Concentration of MDA was determined from standard curve of 1,1,3,3-tetraethoxypropane (a form of MDA) for the range of 0.3-10 nM.

Determination of GSH

GSH, which is accepted to be an indicator of antioxidant capacity, was measured by modified Ellman method.[32] 1 ml of 0.3 M Na_2HPO_4 and 125 µl dithiobisnitrobenzoate was added to 250 µl of supernatant and then vortexed. The mixture was incubated at room temperature for 10 minutes. The GSH level is determined spectrophotometrically by measuring the absorption levels at 412 nm. Levels of GSH were calculated according to following formula:

GSH=(Absorbance of sample x 4.23)/Tissue weight

DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY

SOD activity of the samples was determined by using a commercial assay kit (Cayman's Kit 706002, Cayman Chemical Company, MI, USA). Assay was

conducted according to manufacturer's instructions. Assay kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical.

DETERMINATION OF THE TOTAL ANTIOXIDANT CAPACITY (TAC) AND THE TOTAL OXIDANT STATUS (TOS)

Both parameters were determined by using a commercial kit (Rel Assay Diagnostic, Gaziantep, Turkey). Assays were conducted according to manufacturer's instructions.

TAC assay is based on the reduction of colored 2.2'-azino-bis radical to a colorless reduced form by the antioxidants in the samples. Absorbance of samples was measured spectrophotometrically at 660 nm, and the results were calibrated with the Trolox equivalent (a vitamin E analog), and were expressed in mmol/L.

TOS Assay is based on the principle that oxidants in the sample can oxidize the ferrous ion chelator complex to ferric ion. Ferric ions form a colored complex with a chromogen in an acidic medium. The color intensity which is measured spectrophotometrically at 530 nm is related with the total amount of oxidant molecules in the sample. Calibration was performed with H_2O_2. Results were expressed in μmol H_2O_2 Eq/L.

CALCULATION OF OXIDANT SYSTEM INDEX

Oxidant system index (OSI) is an indicator of the intensity of oxidant damage, and it is a unitless parameter calculated by the ratio of TOS value to TAC value.[33-35]

OSI=TOS/TAC

STATISTICS

The data obtained from the experiments were analyzed using Sigma Stat statistical software. One-way ANOVA test was used for the analysis of variance between groups. Nonparametric Student's t-test was used for paired comparison. $p<0.05$ was considered as significance level. Results were expressed as mean ± standard error.

RESULTS

LEVELS OF MDA

Compared with the control group, MDA levels decreased in all hypoxic groups (Figure 1); decrement in PC and SH groups were more notable ($p<0.05$). MDA levels in SH and PC+SH groups gradually increased compared with the PC group ($p<0.05$) (Figure 1).

LEVELS OF TOS

Since several ROS contribute to oxidative stress processes, measurement of TOS is more useful to evaluate overall oxidative state of tissue. Compared with the control group, TOS levels increased significantly in the PC+SH group ($p<0.05$) (Figure 2).

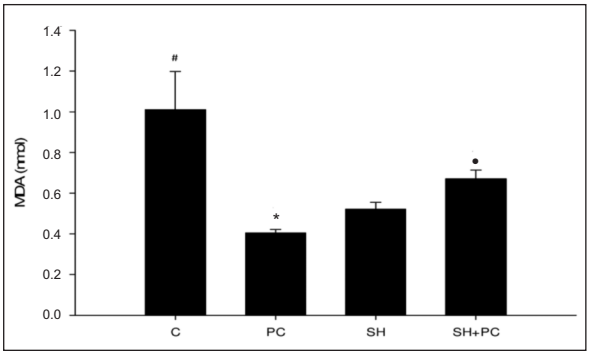

FIGURE 1: MDA Levels in Lung Tissue. MDA Level is significantly higher in control group. (# C vs PC, C vs SH $p<0.05$; * PC vs SH, PC vs PC+SH $p<0.05$; • SH vs PC+SH= $p<0.05$; C vs PC+SH $p=0.085$).

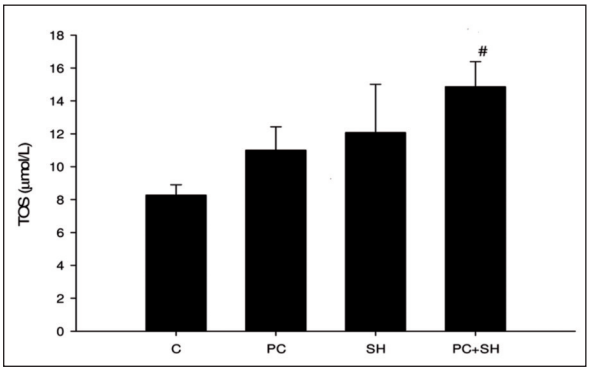

FIGURE 2: TOS Levels in Lung Tissue. TOS levels significantly increased in the PC+SH group (C vs PC+SH $p<0.005$).

LEVELS OF OSI

OSI is an important indicator of oxidative stress as it shows the state of equilibrium between pro-oxidants and antioxidants. Compared to other groups, OSI of PC+SH group was significantly higher ($p<0.05$) (Figure 3).

LEVELS OF TAC

Even though there was no significant difference between the groups, TAC showed a decreasing trend in PC+SH group compared to other groups ($p=0.072$) (Figure 4).

LEVELS OF SOD

SOD is an important antioxidant enzyme that converts the superoxide anion to hydrogen peroxide (H_2O_2), and its levels also indicates the status of superoxide anion formation, and helps for the estimation of oxidative stress. In the present study, SOD activity was increased in control group ($p<0.05$). Compared to other groups, SOD activity decreased significantly in the PC + SH group ($p<0.05$) (Figure 5).

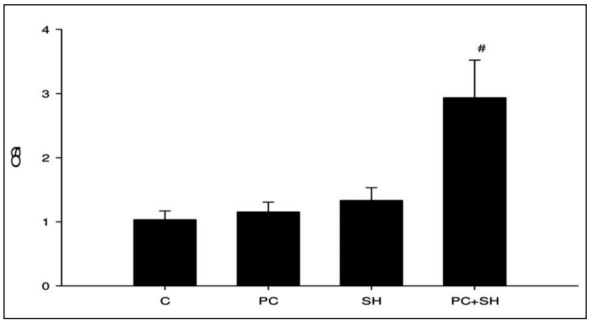

FIGURE 3: OSI Levels in Lung Tissue. OSI was significantly higher in PC+SH (PC+SH vs C, PC+SH vs PC, PC+SH vs SH $p<0.05$).

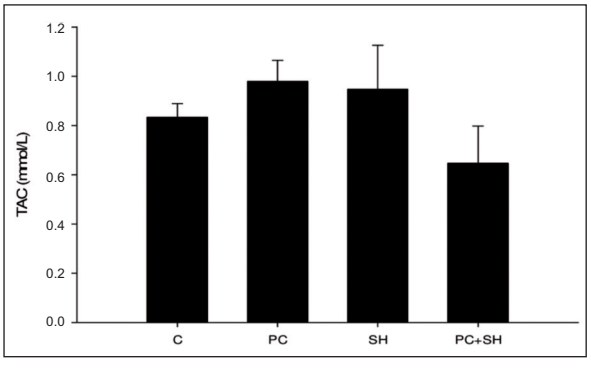

FIGURE 4: TAC Levels in Lung Tissue. PC+SH vs PC $p=0.072$.

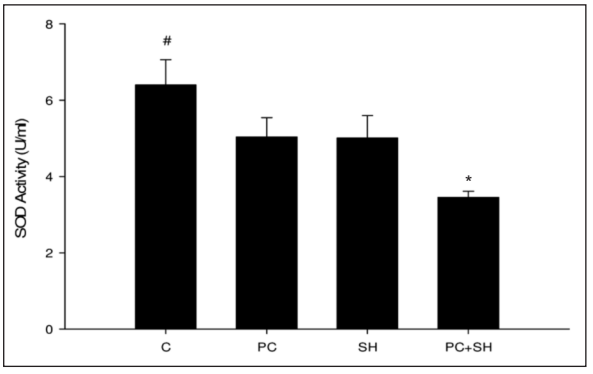

FIGURE 5: SOD Levels in Lung Tissue. SOD activity was significantly higher in control group, and it was decreased in PC+SH group (# C vs PC+SH=$p<0.05$; * PC+SH vs PC, PC+SH vs SH $p<0.05$).

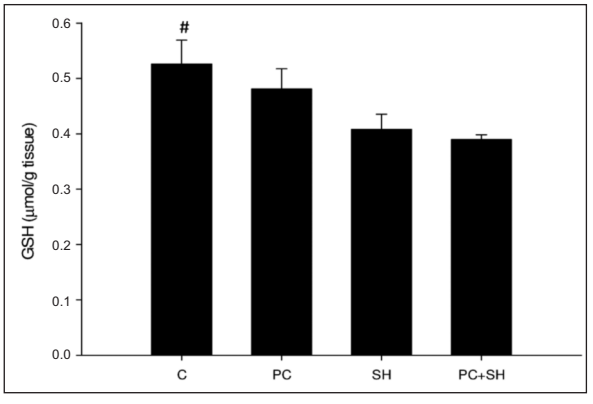

FIGURE 6: GSH Levels in Lung Tissue. In control group GSH levels were significantly higher compared to other groups. (C vs SH $p=0.056$; C vs PC+SH $p<0.05$; PC vs PC+SH $p=0.05$).

LEVELS OF GSH

GSH is an important antioxidant that is found in vast amounts in lung epithelial cells and epithelial lining fluid. It is involved in the reduction of H_2O_2 and lipid hydroperoxides. GSH levels decreased significantly in PC+SH group compared to both C and PC group ($p<0.05$) (Figure 6). Furthermore, a decreasing trend in SH group has been seen compared to C group ($p=0.056$) (Figure 6).

DISCUSSION

Hypoxia changes the redox balance in cells and causes oxidative stress. Hypoxic preconditioning has been shown to be protective against severe hypoxia or ischemia reperfusion damage in several tissues including lung.[13-17] However the preconditioning re-

lated protection is not well defined in the lung tissue. Therefore we aimed to see whether hypoxic preconditioning affects the oxidant-antioxidant balance and improve the antioxidant capacity in lung. Our major finding in this study was hypoxic preconditioning does not improve the antioxidant defense.

Lungs are exposed to both high levels of oxygen and various exogenous and endogenous oxidants because of their nature. Therefore lung tissue is prone to form ROS and reactive nitrogen species (RNS). An increase in the levels of these substances or an imbalance of oxidant / antioxidant system causes oxidative stress that leads to lipid, protein and DNA damage. To overwhelm the deleterious effects of oxidative stress, lungs possess an advanced antioxidant defense system, and in this defense system GSH holds an important place.[36] It is known that ROS and lipid peroxide by products may promote the gene expression of antioxidant enzymes.[37,38] Indeed in our study, MDA levels in control group were higher than the hypoxic groups, and parallel with this finding, antioxidant parameters SOD and GSH levels were also high in control group. As already known, superoxide anion, a ROS, is formed from molecular oxygen, and converted to H_2O_2 by the means of SOD. Therefore increased SOD levels may indicate the increased superoxide anion formation. Moreover TOS and OSI were low in control group.

MDA levels were low in all of the hypoxia groups, as compared to the control group. This decrement was significant in PC and SH groups. However, when compared to the preconditioning group, MDA levels increase in the SH and PC+SH groups; these two groups have higher intensity and longer duration of hypoxia respectively. Even though this result is not consistent with the studies that state hypoxia increases the lipid peroxidation; it is parallel with the studies state that lipid peroxidation followed by oxidative damage increase depending on the severity and duration of hypoxia. In Operation Everest III study, it has been shown that whole blood lipid peroxidation increased parallel to the increasing altitude, by a mean of 23% at 6000 m and by 79% at 8848 m.[36] Nakanishi et al. used a 5500 m hypobaric hypoxia model on rats for 12 hours, 1, 3, 5, 7, 14 and 21 days.[5] They showed that levels of MDA in heart, lungs, kidneys, liver and serum were increased in accordance with the duration of hypoxia, and this increase was maximum on day 21. The researchers also emphasized that increase in MDA levels in the first five days of hypoxia was not uniform in major organs. Low MDA levels in hypoxia groups may be explained by lung tissue being possibly more adaptive to hypoxia compared to other tissues. Indeed, by using a 9000 m and 3 hours of hypoxia model, Mogil'nitskaia et al. showed that lipid peroxidation side products such as diene conjugates and Schiff bases have been decreased with hypoxia while the activity of antioxidant enzymes such as SOD and catalase have been increased in rat lung tissue.[39]

Over the recent years protective effects of hypoxic preconditioning against severe hypoxia damage in various tissues draw attention. Park et al. demonstrated that in brain tissue of neonatal rats hypoxic preconditioning is protective against hypoxic/ischemic damage, and the overall survival time of rats has been increased by hypoxic preconditioning.[40] Berger et al. demonstrated that mild (16%) hypoxic preconditioning reduces the infarct area which forms after ischemia/reperfusion in myocardial cells at similar rates of moderate (12%) and severe (8%) hypoxia. Hypoxic preconditioning in lungs has been shown to reduce hypoxia induced pulmonary vasoconstriction and pulmonary edema by several studies.[4,13,28,41]

The most prominent finding of our study is the increase in TOS, OSI and MDA levels in preconditioning + severe hypoxia group, and this is consistent with the studies stating that hypoxia increases the production of radicals. However, the decrease in antioxidant parameters in preconditioning group and preconditioning+severe hypoxia group are not enough to state that hypoxic preconditioning effects antioxidant capacity. Decrease in the levels of GSH and SOD may possibly be due to their usage in detoxification process of ROS that increases with hypoxia, as well as may be due to structural damage of enzymatic and non-enzymatic antioxidants by hypoxia.

The hypoxic preconditioning model (duration and O_2 level) that used may also be effective in assessing preconditioning effects, in fact, there are studies that suggests that hypoxic preconditioning increases the antioxidant capacity of lungs and reduces oxidative damage.

CONCLUSION

In summary, our findings state that hypoxic preconditioning does not have a significant effect on the antioxidant defense system in the lungs, even though severe hypoxia does effect oxidant/antioxidant systems. To have more obvious results on this issue, transcription factors that trigger hypoxia effects, mitochondrial respiratory chain pathways, cytosolic and mitochondrial ROS and RNS sources which increase by hypoxia, and histological and molecular structures that shows the structural integrity of the cell should be investigated.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

All authors contributed equally while this study preparing.

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