Endothelial Dysfunction Improvement Mechanism By Hyperbaric Oxygen In Sprague Dawley By High-Cholesterol Diet

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Abstract. The purpose of this study was to investigate the mechanism of hyperbaric oxygen could improve endothelial dysfunction. In the experimental method, Sprague Dawley strains divided 3 groups: the normal control for p1, high cholesterol diet for p2, and high cholesterol diet with hyperbaric oxygen of 2.4 ATA with 98% O2 for 3 sessions with the duration of 30 minutes / session, and air break for 5 minutes between each session for the period of 10 days consecutively for p3. On the last day of treatment, serum was taken for heme oxygenase-1 (HO-1), Sirtuin (SIRT1), endothelial Nitric Oxide Synthase (eNOS) and lipoprotein-associated phospholipase A2 (Lp-PLA2) by ELISA method. The new findings of this study are hyperbaric oxygen could improve endothelial dysfunction which occurs due to an atherogenic diet, through two pathways. The first, hyperbaric oxygen therapy increased heme oxygenase1 (HO-1) (p = 0.000), sirtuin-1 (SIRT1) (p = 0.025), endothelial nitric oxide synthase (eNOS) (p = 0.000) and decreased levels of lipoprotein-associated phospholipase A2 (Lp-PLA2) (p = 0.000). The second hyperbaric oxygen administration enhances sirtuin1 (SIRT1) (p=0.000) directly, endothelial nitric oxide synthase (eNOS) (p = 0.000) and decreased levels of lipoprotein-associated phospholipase A2 (Lp-PLA2) (p = 0.000).

Keyword : Endothelial dysfunction, Hyperbaric Oxygen (HBO), High Cholesterol Diet

1. Introduction

Endothelial dysfunction is a disorder of endothelial function due to an imbalance between contraction and relaxation factors. This dysfunction causes endothelial failure to release NO as a mediator of NO vasodilatation. Endothel has an important role in maintaining blood vessel integrity. In normal circumstances, the mediators released are NO and prostacyclin. Nitric Oxide (NO) does not only works for vascular smooth muscle dilatation but also play a role in inhibiting smooth muscle proliferation, platelet aggregation, inhibiting platelet adhesion on endothelial surfaces [1]. In addition, NO is also anti-inflammatory agents by inhibiting monocyte adhesion and neutrophils on endothelial surfaces and antioxidants. Nitric Oxide (NO) is produced by endothelial Nitric Oxide Synthase (eNOS) enzymes. The presence of platelet adhesion, monocytes, neutrophils on the endothelial surface is the initial process of atherosclerosis [2].

Hyperbaric oxygen therapy is a type of treatment where the patient breathes with 100% oxygen through a mask and is at a pressure of more than one ATA (2.4 ATA) within a certain period of time. Hyperbaric oxygen therapy is based on the role of Reactive Oxygen Species (ROS) molecules in signaling molecules in the transduction cascade for various transcription factors, growth factors, cytokines, and hormones [3].

One of the gene products is caused by oxidants that play an important role in adaptive and protective responses to oxidative stress is heme oxygenase-1 (HO-1) which is a protein that protects cells from
oxidative stress and Sirtuin (SIRT1) which acts as an anti-inflammatory endothelial cell, prevents the formation of foam cells and is antiatherogenic and cardioprotective cells so that SIRT1 has a very promising role for endothelial dysfunction and atherosclerotic therapeutic approaches [4].

2. Experimental Methods
The research was conducted at the Faculty of Veterinary Medicine, Airlangga University of Surabaya. The sample animals are rats of Sprague Dawley strain, weighing about 150 grams. Endothelial dysfunction is made by giving a high cholesterol diet with a special formula [4]. The hyperbaric oxygen was administered at a dose of 2.4 ATA for 10 days consecutively. This study was conducted for 73 days with the following timing: adaptation period for 14 days, high cholesterol diet for 49 days and administration of hyperbaric oxygen (HBO) for 10 days. High cholesterol diet was administered with the composition of modification formula containing sodium acids via sonde to each rat/day and high cholesterol diet with mixed compositions of comfeed PAR-S, flour, cholesterol, pork oil, starch, and water, which were made into pellet then dried. The high cholesterol diet is a formulation of a preliminary study carried out by examining the lipid profile and examining the level of lipoprotein-associated phospholipase A2 (Lp-PLA2) as a marker of endothelial dysfunction.

The followings are the division of groups: group 1 (p1): male Rattus norvegicus group of Sprague Dawley strains which are given a normal diet; group 2 (p2): male Rattus norvegicus group of Sprague Dawley strains which are given an high-cholesterol diet; group 3 (p3): male Rattus norvegicus group of Sprague Dawley strains which are given an high-cholesterol diet and given hyperbaric oxygen 2,4 ATA for 10 days consecutively.

The activity of SIRT 1 in all three groups of rats are examined using Elisa methods. From the experimental results, the data were analyzed by SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL).

3. Results and Discussion
The data of this study had 3 treatments groups namely normal control group (p1), negative control group (atherogenic diet) (p2) and treatment group (atherogenic diet + HBO) (p3). During the research there are 4 variables employed used 4 variables: Lipoprotein-associated phospholipase A2 (Lp-PLA2), Heme oxygenase 1 (HO-1), Sirtuin 1 (SIRT 1) and endothelial nitric oxide synthase (eNOS).

The result of multiple comparisons with Tukey HSD
HO 1 results were not significant in all treatment groups. While In SIRT-1 and LpPLA 2, there were significant differences in all groups (normal control, negative control (atherogenic diet) and treatment group (atherogenic diet + HBO), while. It can be seen in Table 1

| Groups                          | Normal Control (P1) | Negatve Control / atherogenic diet (P2) | Treatment (atherogenic diet + HBO) (P3) |
|--------------------------------|---------------------|----------------------------------------|----------------------------------------|
|                                | Mean (0,122±0,029)  | Mean (0,106±0,037)                      | Mean (0,171±0,031)                      |
| Normal Control (P1)            |                     |                                        |                                        |
| Negative Control / atherogenic diet (P2) | 0,504               | -                                      | 0,006*                                 |
| Treatment (atherogenic diet + HBO) (P3) | 0,006*               | 0,000*                                |                                        |

Note: * significant at α level=0,05

The results of the analysis showed a significant level in the normal control group compared to the treatment group (p = 0.006), the negative control group compared to the treatment group (p = 0,000).
Table 2. Result of multiple comparisons from Sirtuin 1 (SIRT 1)

| Groups                                      | Normal Control (P1) | Negative Control / atherogenic diet (P2) | Treatment (atherogenic diet + HBO) (P3) |
|---------------------------------------------|---------------------|------------------------------------------|----------------------------------------|
| Mean (Normal Control (P1))                  | (0.188±0.017)       | Mean (P2)                                | Mean (P3)                              |
| Mean (P1)                                   |                     | Mean (0.114±0.029)                       | Mean (0.252±0.027)                      |

- Normal Control (P1)  
- Negative Control / atherogenic diet (P2)  
- Treatment (atherogenic diet + HBO) (P3) 

| Groups                                      | Normal Control (P1) | Negative Control / atherogenic diet (P2) | Treatment (atherogenic diet + HBO) (P3) |
|---------------------------------------------|---------------------|------------------------------------------|----------------------------------------|
| Mean (Normal Control (P1))                  | (0.188±0.017)       | Mean (P2)                                | Mean (P3)                              |
| Mean (P1)                                   |                     | Mean (0.114±0.029)                       | Mean (0.252±0.027)                      |

From the calculation analysis results, it can be seen that significant level there were in all groups (p = 0.000).

Table 3. Result of multiple comparisons from lipoprotein-associated phospholipase A2 (Lp-PLA2)

| Groups                                      | Normal Control (P1) | Negative Control / atherogenic diet (P2) | Treatment (atherogenic diet + HBO) (P3) |
|---------------------------------------------|---------------------|------------------------------------------|----------------------------------------|
| Mean (Normal Control (P1))                  | (0.215±0.054)       | Mean (P2)                                | Mean (P3)                              |
| Mean (P1)                                   |                     | Mean (0.441±0.102)                       | Mean (0.185±0.042)                      |

- Normal Control (P1)  
- Negative Control / atherogenic diet (P2)  
- Treatment (atherogenic diet + HBO) (P3) 

From the calculation analysis results, it can be seen that significant level there were in all groups (p = 0.000).

Table 4. Result of multiple comparisons from endothelial nitric oxide synthase (eNOS)

| Groups                                      | Normal Control (P1) | Negative Control / atherogenic diet (P2) | Treatment (atherogenic diet + HBO) (P3) |
|---------------------------------------------|---------------------|------------------------------------------|----------------------------------------|
| Mean (Normal Control (P1))                  | (0.221±0.030)       | Mean (P2)                                | Mean (P3)                              |
| Mean (P1)                                   |                     | Mean (0.199±0.048)                       | Mean (0.422±0.024)                      |

- Normal Control (P1)  
- Negative Control / atherogenic diet (P2)  
- Treatment (atherogenic diet + HBO) (P3) 

From the calculation analysis results, it can be seen that significant level there were in all groups (p = 0.000).
Relationship Between Variables in the Study Groups (Lipoprotein-associated phospholipase A2 (Lp-PLA2), Heme oxygenase 1 (HO-1), Sirtuin 1 (SIRT1) and endothelial nitric oxide synthase (eNOS)).

The relationship between variables (lipoprotein-associated phospholipase A2 (Lp-PLA2), Heme oxygenase 1 (HO-1), Sirtuin 1 (SIRT1) and endothelial nitric oxide synthase (eNOS)) was analyzed using regression analysis. Regression analysis was conducted to determine the causal relationship between the variables of the study (lipoprotein-associated phospholipase A2 (Lp-PLA2), Heme oxygenase 1 (HO-1), Sirtuin 1 (SIRT1) and endothelial nitric oxide synthase (eNOS)) and the causes of changes in research variables. Determination of the variables used in the regression analysis used the consideration of the analysis of dependent variables, as well as the basic theory of this research. Positive values indicate that if the independent variable increases, the dependent variable will also increase as value b. Negative values indicate that if the independent variable decreases, the dependent variable will also decrease as value b.

Table 5. Causal Relationship, a value of standardized coefficient (b) and significance between variable on pathway analysis

| Variable               | Value of standardized coefficient (b) | Significance (p) |
|------------------------|---------------------------------------|------------------|
| HBO → HO-1             | (+) 0.650                              | 0.000            |
| HO-1 → SIRT1           | (+) 0.220                              | 0.025            |
| HBO → SIRT1            | (+) 0.901                              | 0.000            |
| SIRT-1 → eNOS          | (+) 0.812                              | 0.000            |
| eNOS → Lp-PLA2         | (-) 0.819                              | 0.000            |
| Atherogenic Diet → Lp-PLA2 | (+) 0.676                           | 0.000            |

Based on Table 5: positive values indicate that if the independent variable increases, the dependent variable will also increase. The administration of hyperbaric oxygen (HBO) will increase the levels of heme oxygenase-1 (HO-1), then levels of heme oxygenase-1 (HO-1) will increase levels of Sirtuin-1 (SIRT1). Hyperbaric oxygen (HBO) administration also directly increases SIRT1 levels, the increase in levels of Sirtuin-1 (SIRT1) will increase levels of eNOS. Negative values indicate that increasing eNOS will inhibit the level of the enzyme lipoprotein-associated phospholipase A2 (Lp-PLA2).

On the results of pathway analysis due to hyperbaric oxygen (HBO) administration, the final path analysis is obtained as described below:

![Figure 1 Final Pathway Analysis](image-url)
The results of the analysis in Table 5 and Figure 1 shows that from this study there were two significant pathways due to hyperbaric oxygen (HBO), namely:

1. HBO $\rightarrow$ HO-1 $\rightarrow$ SIRT1 $\rightarrow$ eNOS $\rightarrow$ Lp-PLA2
2. HBO $\rightarrow$ SIRT1 $\rightarrow$ eNOS $\rightarrow$ Lp-PLA2

The dominant pathway due to hyperbaric oxygen (HBO) administration is obtained through calculating by multiplying the standardized coefficient values of the variables that have a significant relationship as follows:

1. HBO $\rightarrow$ HO-1 $\rightarrow$ SIRT1 $\rightarrow$ eNOS $\rightarrow$ Lp-PLA2
   
   $0.650 \times 0.220 \times 0.812 \times -0.819 = -0.095$

2. HBO $\rightarrow$ SIRT1 $\rightarrow$ eNOS $\rightarrow$ Lp-PLA2
   
   $0.901^2 \times 0.812 \times -0.819 = -0.539$

So it can be concluded that the second path that is without going through HO-1 first is a stronger path than the first step through HO-1, before going to Sirtuin-1 (SIRT1). In this study, hyperbaric oxygen exposure to endothelial dysfunction resulting from atherogenic diets will lead to several signaling processes in cells, such as Heme oxygenase 1 (HO-1), Sirtuin 1 (SIRT1) and endothelial nitric oxide synthase (eNOS) and inhibition of lipoprotein-associated phospholipase A2 (Lp-PLA2), all of which influence each other that shows the improvement of endothelial dysfunction. In the series of signaling processes that occur, it is preceded by the activation process of transcription factors such as NFκB due to an increase in ROS molecules from hyperbaric oxygen exposure, although NFκB was not examined in this study. The transcription factor will determine gene expression that functions in adaptative and cell protection, including proteins that are also antioxidants such as heme oxygenase-1 (HO-1) and other proteins such as sirtuin (SIRT1) which are cardioprotective.

Administration of HBO can also induce the release of nitric oxide (NO), NO molecules have a role in the regulation of endothelial cells through the path of Extracellular Signal-Regulated Kinase (Gu, Lynch and Brecher, 2000). Biomolecular effects of hyperbaric oxygen exposure (HBO) through increased ROS molecules produced will affect the activation of NFkB transcription factors that will produce cell adaptive responses, proteins that act as antioxidants and have protective effects on endothelial dysfunction are HO-1, SIRT1, and eNOS.

The chemical reactivity of high ROS molecules allows cells to form scavenger molecules, antioxidants that will maintain redox homeostasis in cells, by increasing the expression of antioxidant genes that will reduce the level of oxidative stress. ROS molecules also activate several transcription factors such as NFkB to express proinflammatory proteins (Silva, Pernomian and Bendhack, 2012). ROS molecules formed after HBO exposure will modulate adaptative functions that protect cell damage [5].

Basically ROS that occurs because of hyperbaric oxygen therapy (HBO) is mostly hydrogen peroxide (H2O2) (Thom, 2011). If the cell is exposed to ROS, then the cell does not immediately experience death. However to prevent damage caused by ROS, the cell has the ability to reduce ROS, which is through the enzyme scavenger system, including Super Oxide Dismutase (SOD), which acts to change hydrogen peroxide (H2O2) becomes water and oxygen [6].

The administration of HBO must still pay attention to the dose and interval of administration because the appropriate dose is very important. Remembering if there is excessively the ROS molecules will cause the adaptive function of the cell to be unable to compensate for the free radical overflow and will cause a state of oxidative stress in the cell, being unable to equilibrate the overflowing and will cause adverse effects for cells.

The principal of the hyperbaric oxygen therapy (HBO) are the concept of redox hypotheses, hormesis and hypoxia precondition, those are:

1. Redox hypothesis: taking the advantage of the arisen Reactive Oxygen Species (ROS).
2. Hormesis: the dose is associated with the production of H2O2 which is an oxidant stress in physiological condition. The oxidant eustress is between 0-10 nanomolar, hormesis therapy uses an appropriate dose of adaptive response which is range between 10-100 nanomolar, whereas the dose>100 nanomolar should be avoided because of causing oxidative stress. However each disease is different depending on the microenvironment.
3. Hypoxia precondition: having response that corresponds to HIF 1 alpha. However in particular several conditions, it can change according to the microenvironment [7].
The results of this study show that administration of HBO can improve endothelial dysfunction in normal individuals and there is a balance between oxidants and antioxidants in the body. So that the stimulation and activation of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, have significant changes through HBO therapy. Based on the theory of Window Period For Oxidative Stress Attenuating Intervention (WPOS). This is because the period of giving antioxidants will give positive results in cells that experience oxidative stress. It is between normal oxidative stress and disease caused oxidative stress levels, which are called pseudo-health oxidative stress levels [8].

This study, uses the doses that have been previous in the study. The dose is important because it can show a different biological response, which can be explained by the hormesis theory. It is a process that uses exposure the low doses of chemicals or damaging environmental factors at the higher doses, which can induce the advantage adaptively on cells or organisms [9]. The mechanism of hormesis involves the activation of adaptive cellular stress response pathways which are involving kinase receptors and activation of transcription factors that induce cytoprotective protein expression such as antioxidant enzymes, chaperone, and growth factors, [9].

Hyperbaric oxygen (HBO) administration causes an increasing in Reactive Nitrogen Species (RNS) and reactive oxygen species (ROS). Reactive oxygen species (ROS) that produced by HBO is hydrogen peroxide (H2O2), which is then changed into water (H2O) by the enzyme catalase and glutathione peroxidase [10]. Air breaks for 5 minutes use ordinary air in not only to preventing oxygen toxicity, but also to create relative hypoxic conditions that cause a state of ischemic precondition. At 2.4 ATA, administration of hyperbaric oxygen (HBO) cause oxygen saturation of 5.39. If the administration of pure oxygen is stopped and replaced with ordinary air, so the oxygen saturation will decrease to 1.01 which is likely to be hypoxic. This relative hypoxia will activate the transcription factor hypoxia-inducible factor 1 (HIF-1). As a adaptive response to cellular stress.

Free radicals such as ROS that produced during hyperbaric oxygen (HBO) treatment, are considered safe. Hyperbaric oxygen (HBO) treatment pressure is never more than 3 ATA and usually does not occur more than 90 minutes. It is because of the adaptive response of the cell by looking at the main physiological benefits of adaptive response, which is to protect or retain cells and organisms from toxic substances. Adaptive responses are induced by oxidative stress [11]. A short exposure to HBO therapy showed adequate antioxidant defenses so biochemical stress due to increasing ROS was still relevant. ROS increases in organs that experience hyperoxia, then antioxidants against excess ROS. There are two types of antioxidants, enzymatic antioxidants such as superoxide dismutase, catalase, thioredoxin, glutathione peroxidase, and reductase, while non-enzymatic ones such as vitamin C, vitamin E, B-carotene and carotene (Thom, 2011).

The impact of protection from the use of HBO can be mediated by antioxidant enzymes that are directly involved in preventing oxidative cell damage and repair enzymes that can eliminate or repair oxidatively damaged macromolecules. Free radicals in the tissue will be offset by Superoxide Dismutase (SOD) to prevent tissue injury which is an antioxidant defense system. HBO treatment can cause an antioxidant mechanism and reduce oxidative stress [11].

In HBO therapy there is an increase in oxygen transfer to the tissues through two mechanisms. The first is the increase of hemoglobin saturation by 20.1% [12]. Oxygen in the blood is transported in soluble form in plasma fluid and forms a bond with hemoglobin and only a small portion (3%) is found in the soluble form. Oxygen in this soluble form will be very important in this therapy because the nature of dissolved oxygen will be more easily consumed by the tissue through direct diffusion than oxygen bound to hemoglobin [3].

There are two pathway of the mechanism of study. The first pathway of the mechanism starts with hyperbaric oxygen therapy (HBO) which provides benefits from ROS and RNS [10]. Reactive Oxygen Species (ROS) triggers proteins released by cells as a response to oxidative stress: heme oxygenase-1 (HO-1) [13] which will induce SIRT1 [14]. The second pathway, ROS triggers SIRT1 [15]. Furthermore, both of the first and second pathways, Sirtuin will induce eNOS, the enzyme that converts L arginine to NO and L citrulline [16,17]. Nitric Oxide (NO) prevents LDL oxidation which is the main player with a variety of significant antagonist roles and is involved in all phases of atherogenesis [18].
4. Conclusion
There are two mechanisms of repairing 2.4 ATA post hyperbaric endothelial dysfunction (HBO) with 98% oxygen 3x30 minutes/session with 5 minutes water break period for 10 consecutive days, in animals exposed to atherogenic diets occur through two pathways. The first pathway is that administration HBO increases heme oxygenase (HO-1), sirtuin-1 (SIRT1), endothelial nitric oxide synthase (eNOS) and decreased levels of the enzyme lipoprotein-associated phospholipase A2 (Lp-PLA2). The second pathway is the administration of HBO directly increased sirtuin-1 (SIRT1), endothelial nitric oxide synthase (eNOS) and decreased levels of the enzyme lipoprotein-associated phospholipase A2 (Lp-PLA2).

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