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Research Article

The Effect of HBOT on SIRT-1 and SYNDECAN-1 as Therapeutic Targets for Endothelial Dysfunction

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ABSTRACT

Background: The effect of HBO_2 alone on Sirt-1 and Syndecan-1 is unknown, even though both molecules are involved in preventing endothelial dysfunction. This study aims to determine the effect of HBO_2 on Sirt-1 and Syndecan-1 as therapeutic targets for endothelial dysfunction. **Method:** This study employed a true experimental post-test design. Twenty male Sprague Dawley rats aged 12-14 weeks were divided into two groups. Diving was carried out in an animal hyperbaric chamber with a dose of 2.4 ATA for 60 minutes. All data were collected 18 hours after diving. **Results:** Our study revealed that the administration of HBO caused an increase in serum MDA and endothelial NF-kB levels ($p = 0.007$; $p = 0.001$, respectively) without an increase in any inflammatory markers, specifically IL-1 and VCAM-1 levels ($p = 0.707$; $p = 0.168$, respectively). HBO_2 decreased Syndecan-1, a marker of endothelial injury ($p = 0.026$), but did not affect endothelial eNOS and Sirt-1. **Conclusion:** HBO_2 did not cause endothelial injury and inflammation, but the dose used was not enough to increase Sirt-1 levels. Additional research is needed to determine a hormesis dose that can increase Sirt-1 levels.

Keywords: Sirt-1, Syndecan-1, HBO_2, Endothelial dysfunction.

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BACKGROUND

Endothelial dysfunction describes the transition from a normal and healthy endothelium to a damaged or stressed endothelium characterized by a pro-vasoconstriction, pro-coagulation, and pro-inflammatory phenotype.[1] In diving, inflammation and endothelial dysfunction are considered contributing factors to decompression sickness triggered by bubbles.[2-5]

The luminal side of the blood vessel endothelium is covered by the glycocalyx, which consists of proteoglycans, glycosaminoglycans, and glycoproteins. The glycocalyx serves as a crucial regulator of mechanotransduction, thrombosis, inflammation, vascular permeability, and cytokine signaling due to its interactions with circulating cells, such as immune cells.[6-8] Syndecan-1 is a proteoglycan component that has been extensively investigated in its association with mechanosensory shear stress in endothelial cells[9], regulating the differentiation of vascular smooth muscle cells[10], and endothelial inflammatory mediators.[11] Elevated circulating levels of Syndecan-1 indicate degradation of the endothelial glycocalyx, which is linked to increased mortality, coagulopathy, and inflammation.[12]

One protein that has received attention regarding its involvement in endothelial dysfunction is Sirtuin-1. Sirt-1 is expressed in large quantities in endothelial cells and is the sole member of the Sirtuin family that exhibits unique regulation of endothelial cell physiology, including enhancing the vasodilator and regenerative function of the blood vessel wall through modulating the activity of eNOS, FOXO1, p53, and angiotensin II (Ang II) type 1 receptor (AT1R).[13] Sirt-1 also modulates monocyte adhesion and foam cell formation by regulating VCAM-1 and ICAM-1 expression[14], prevents hydrogen peroxide-induced endothelial cell death through deacetylation of the tumor suppressor p53, and protects blood vessels from endothelial dysfunction-induced hyperglycemia through mechanisms that involve reducing the expression of p66Shc[15] and regulating eNOS through deacetylation of lysine 496 and 506.[16]

Hyperbaric oxygen therapy (HBO_2) involves the use of 100% oxygen in a high-pressure chamber to treat diving-related injuries such as decompression sickness or other clinical conditions such as gangrene.[17] HBO_2 has mechanical effects that can reduce air bubbles[18-20] and biomolecular effects, including anti-inflammatory[21,22] and antioxidant induction.[23,24] Due to these effects, HBO_2 has also been studied as a preconditioning method.[24,25]

HBO_2 has been proven to reduce the number of bubble nuclei in endothelial cells, which are believed to be the origin of bubble formation during diving.[26] However, it has never been studied how HBO_2 affects the glycocalyx layer, the outermost part of endothelial cells.

Protection of this layer has been proven to protect endothelial cells from mechanical damage, including bubbles.[27] Mechanical injury to the glycocalyx layer, as indicated by increased circulating Syndecan-1, leads to increased adhesion of immune complexes, complement activation, and intravascular coagulation.[28-31] Syndecan-1 (CD138) is part of the endothelial proteoglycans involved in various cellular processes, including migration, differentiation, and proliferation. Syndecan-1 can act as a pro- and anti-inflammatory molecule, depending on whether it is present on the cell surface or dissolved in the circulation.[8]

Hyperoxic conditions in HBO_2 lead to elevated production of reactive nitrogen species (RNS)

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and reactive oxygen species (ROS).[2,32] ROS can cause damage to deoxyribonucleic acid (DNA), proteins, lipid membranes, and other cellular components[33], and the products of lipid peroxidation can serve as markers for oxidative stress.[34] The initial signaling induced by ROS includes the activation of transcription factors and post-translational proteins, including mitogen-activated protein kinase (MAPK), nuclear factor kappa beta (NF-κB), the heat shock response, the p53 pathway, phosphoinositide 3-kinase (PI(3)K/Akt), and FOXO. ROS can function as secondary or tertiary messengers in the activation of these redox-sensitive pathways.[35] HBO_2 induces NF-κB activation in human PBMCs, occurring four hours after HBO2 administration, which is a crucial step in HBO_2 treatment.[36]

NF-κB responds to cytokines, toxins, and stress, resulting in inflammatory processes, cellular adaptation and apoptosis.[37] The activation of NF-κB is influenced by the cell's redox status, which affects the phosphorylation of serine 19 and 23 and the inhibitory subunit (IκB) of NF-κB.[38] The cytokine IL-1 can activate NF-κB through the IL-1 receptor (IL-1R) and TLRs. These pathways activate many genes involved in the inflammatory response, such as VCAM-1.[39]

The oxidative stress induced by HBO_2 triggers the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), one of the endothelial cell protection genes.[40,41] Nrf2 is also involved in the neuroprotective effects of Sirtuin-1 (Sirt-1) in preconditioned HBO_2 for a cerebral ischemia model.[42] HBO_2 also prevents endothelial dysfunction in mice with dyslipidemia by increasing Sirt-1.[43] Sirt-1 is expressed in large amounts in endothelial cells, and its levels fluctuate according to cellular processes. It is the only member of the sirtuin family that has been demonstrated to regulate endothelial cell physiology through deacetylation of histone and non-histone proteins, including enhancing the vasodilator and regenerative function of blood vessel walls by modulating the activity of eNOS, FOXO1, p53, and angiotensin II (Ang II) type 1 receptor (AT1R).[13,44,45,16,15] Endothelial cell Sirt-1 and eNOS are regulated through positive feedback resulting in resistance to oxidative stress.[46] NO generated by eNOS reduces the hydrophobicity of the endothelial wall and maintains endothelial integrity, thereby reducing the number of nuclei[47,48], decreasing bubble formation, and reducing the severity of tissue injury.[49,50]

The enzymatic activity of Sirt-1 is controlled directly and indirectly by ROS.[51] Although it has been proven that inhibition of Sirt-1 causes an increase in ROS levels, the mechanism underlying the reduction in ROS by Sirt-1 remains unclear.[46] Antagonistic crosstalk occurs in the NF-κB and Sirt-1 pathways in regulating ROS in endothelial cells. Sirt-1 inhibits NF-κB directly by deacetylating the RelA/p65 subunit at lysine 310 or by activating AMPK and peroxisome proliferator-activated receptor alpha (PPARα), which inhibit the NF-κB pathway, thereby reducing ROS formation.[52] Meanwhile, NF-κB suppresses Sirt-1 activation through the generation of ROS. Thus, Sirt-1 regulates NF-κB signals to control ROS production, and NF-κB reduces Sirt-1 levels to increase ROS production.[46] Sirt-1 also modulates foam cell formation and monocyte adhesion by regulating the expression of VCAM-1 and ICAM-1.[14] Whether this modulation occurs through NF-κB still requires further research.

The mechanism involved in the use of HBO_2 in preventing endothelial dysfunction in diving is not yet defined. Research on the Sirt-1/eNOS and Sirt-1/NF-κB pathways related to the administration of hyperbaric therapy has not been fully explored, and the condition of the glycocalyx after HBO_2 administration has never been studied before. This study aims to examine the effect of HBO_2 on endothelial injury, oxidative stress, and subsequent inflammation through Syndecan-1, MDA, VCAM-1, and IL-1 serum and endothelial NF-κB. This study also analyzes the effect of HBOT on endothelial Sirt-1 and eNOS, which are considered factors in preventing endothelial dysfunction.

METHODS

This study employed a true experimental post-test design. Twenty male Sprague Dawley rats aged 12-14 weeks were divided into pre- and post-diving groups (n = 10). The diving was conducted in an animal hyperbaric chamber at the UPN Veteran Jakarta animal hyperbaric lab. Serological examinations were carried out at PSSP IPB, and IHK analyses were conducted at iRatLab Bogor.

The pre-diving group was terminated before the study began to obtain baseline values, and the post-diving group was terminated 18 hours after the administration of HBO_2. HBO_2 was administered at 2.4 ATA for 60 minutes.

Experimental animals were provided with unlimited access to pelleted rodent food and water. The room lighting was regulated on a cycle of 12 hours of light and 12 hours of darkness. The temperature was maintained at 23-25 °C, and the relative humidity ranged from 50.0% to 56.0%.

Serum Processing

Rats were anesthetized with 0.3 ml of ketamine intramuscularly until they became unconscious. Blood was drawn intracardially using a 3cc syringe, placed in a plain vacutainer, and left at room temperature for 30 minutes before centrifugation. Blood was centrifuged at 3,000 rpm for 15 minutes to isolate serum and stored at -20 °C.

Serum testing was carried out at the Animal Research Laboratory of the Center for Primate Animal Studies (PSSP) of IPB University. Syndecan-1, VCAM-1, IL-1α, and Sirt-1 were examined using immunoenzymatic ELISA according to the manufacturer's instructions. MDA was examined using the colorimetric method (TBA method).

All kits used were sourced from Elabscience, Texas, USA: Rat Syndecan-1 (E-EL-R0996), VCAM-1/CD106 (E-EL-R1061), IL-1α (E-EL-R0011), Sirt-1 (E-EL-R1102), and MDA (E-BC-K023-M). The absorbance was read at 450 nm, and the concentration was determined by comparing the optical density with a standard curve.

Tissue Processing

After blood collection, the pulmonalis was immediately stored in a bottle containing a 10% formalin solution. On the same day, it was delivered to iRatLab Bogor for preparation and immunohistochemistry assays.

The first step involved tissue processing (dehydration, clearing, and embedding). The tissue was fixed and cut into 0.3-0.5 cm thick sections, then dehydrated using a Thermo Scientific™ machine (STP 120 Spin Tissue Processing type). The samples were automatically transferred from alcohol solutions with graded concentrations: 70% alcohol (1 hour), 80% (1 hour), 90% (1 hour), 95% (1 hour), 100% alcohol I (1 hour), 100% II (1 hour), and 100% III (1 hour). The clearing process was carried out with xylol: Xylol I (45 minutes), Xylol II (45 minutes), and Xylol III (45 minutes). Next, liquid paraffin was infiltrated into the tissue using paraffin I, II, and III at a temperature of 60 °C for 30 minutes each in a Thermo Scientific™ machine. The embedding was conducted using a Sakura Tissue TEK III model 4584 tissue embedding machine. After that, liquid paraffin was applied, and the paraffin block was cooled overnight. The paraffin blocks were cut using a Leica brand microtome type RM2235 microtome with a thickness of 4-5 μm, and preparations were made. Before staining, the preparations were incubated at a temperature of 37 °C. Special slides for immunohistochemical staining were coated with poly-l-lysine (Sigma P8920).

All IHC staining kits were sourced from Abcam, Cambridge, UK: Anti-NOS3 Antibody (A-9) (sc-376751), Anti-SIRT1 Antibody (B-7) (sc-74465), Anti-NFκB p65 Antibody (F-6) (sc-8008), and m-IgG Fc BP-HRP, 50 μg/0.5 ml (sc-525409).

Table 1. Mean levels of studied variables pre- and post-HBO2.

Parameter	Pre HBO	Post HBO	p
MDA	159.65 ± 57.12	252.03 ± 62,22	0.007*
IL-1	72.51 ± 69.22	53.34 ± 10.97	0.707
Syndecan-1	14.98 ± 5.01	10.41 ± 2.50	0.026*
VCAM-1	18.28 ± 4.43	14.51 ± 6.47	0.168
NF-kB Endothel	0.56 ± 0.19	1.04 ± 0.29	0.001*
eNOS Endothel	18.54 ± 9.01	11.45 ± 5.05	0.056
Sirt-1 Endothel	1.46 ± 1.1	1.69 ± 0.99	0.653

Level of significant p < 0.05

IHC reading was performed using a Japanese Nikon Eclipse 80i DS-Fi1 microscope with 400x magnification. The displayed image was analyzed with the J-image application to calculate the unit in percentage.

Data Analysis

The data obtained were analyzed using the Shapiro-Wilk test and displayed as mean ± SD. An independent t-test was employed to analyze the effect of HBO on all the variables studied. The significance level for all statistical analyses was set at $p < 0.05$.

RESULTS

None of the experimental animals displayed any barotrauma or seizures during and after HBO_2 treatment. The results are presented in Table 1.

Inflammation

We selected MDA, NF-κB, IL-1, and VCAM-1 as parameters indicating inflammation. The expression of endothelial MDA and NF-κB showed significant changes. Elevated MDA levels were observed after HBO_2 treatment (252.03 ± 62.22 vs. 159.65 ± 57.12; $p = 0.007$). Similarly, an elevated endothelial NF-κB expression was noted after HBO_2 treatment (1.04 ± 0.29 vs. 0.56 ± 0.19; $p = 0.001$).

Conversely, circulating VCAM-1 and IL-1 levels decreased, although the decrease was not significant. Serum VCAM-1 levels decreased from 18.28 ± 4.43 to 14.51 ± 5.05 ($p = 0.168$), and serum IL-1 levels decreased from 72.51 ± 69.22 to 53.34 ± 10.97 ($p = 0.707$).

Endothelial Dysfunction

Our study showed that the administration of HBO_2 only affected serum levels of Syndecan-1 but did not affect endothelial eNOS and Sirt-1 levels. However, HBO_2 significantly lowered circulating Syndecan-1 levels compared to controls (10.41 ± 2.50 vs. 14.98 ± 5.01; $p = 0.026$).

Although it did not achieve statistical significance, the expression of endothelial eNOS decreased after HBO_2 treatment (14.51 ± 6.47 vs. 18.54 ± 9.01; $p = 0.056$), while the expression of endothelial Sirt-1 slightly increased after HBO_2 treatment (1.69 ± 0.99 vs. 1.41 ± 1.1; $p = 0.653$).

DISCUSSION

Inflammation

An increase in oxygen levels due to HBO_2 leads to elevated dissolved oxygen in serum and tissues (hyperoxia). Hyperoxia causes elevated production of ROS, such as superoxide and peroxynitrite, decreased NO levels, and increased endothelial cell death due to oxidative stress. Serum MDA levels, a product of lipid oxidation, serve as a sensitive marker of endothelial dysfunction.[1]

The increased ROS formation in this study was followed by an increase in the pro-inflammatory transcription factor NF-κB by initiating IκB dissociation, thus resulting in the translocation of NF-κB to DNA chains. Many genes involved in the inflammatory response are activated through this pathway, including MnSOD, iNOS, VCAM-1, inflammatory cytokines (TNF and Interleukins (IL-1, IL-2, IL-6)), inducible COX-2, chemokines, and adhesion molecules.[39]

VCAM-1 is an adhesion factor expressed on activated endothelial cells and can also be found in soluble form in serum. It helps regulate inflammation-related vascular adhesion and trans-endothelial migration of leukocytes, including macrophages and T cells. VCAM-1 can be induced by ROS, interleukin-1 beta (IL-1β), or tumor necrosis factor-alpha (TNFα) produced by various cells.[53] Recent research suggests that VCAM-1 has potential as a marker of endothelial changes. IL-1α is a cytokine serving as a signaling molecule in inflammation, including being one of the first responses to an insult. Cells that produce IL-1 include macrophages, endothelial cells, and epithelial cells. Its increase in serum indicates the presence of a systemic inflammatory process.[54]

However, in this study, the increase in NF-κB was not accompanied by an increase in VCAM-1 and IL-1α. The ability of HBO_2 to reduce VCAM-1 and IL-1α levels is consistent with other research related to the use of HBO_2 as a precondition to prevent endothelial dysfunction in decompression sickness and diabetic foot treatment.[21,55] This indicates that oxidative stress due to HBO_2 treatment is not sufficient to cause endothelial dysfunction and inflammation.

An increase in MDA without being followed by other pro-inflammatory variables suggests that HBO_2 does not cause oxidative stress but oxidative eustress. Oxidative stress results from an imbalance between the elevated formation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) and available antioxidants.[56,57] Oxidative eustress refers to a physiological adaptive oxidative stress response (low level) used in redox signaling and regulation.[58] These results align with other studies stating that hyperoxic conditions resulting from hyperbaric oxygen therapy increase the expression of ROS (MDA)[59-61] without systemic inflammation.[62]

Endothelial Dysfunction

Elevated serum syndecan-1 levels indicate cell damage, inflammation, or activation of endothelial cells. Syndecan-1 is now used as a target in treating inflammation, including in COVID-19 patients.[30] The significant decrease in serum syndecan-1 in this study indicates the absence of endothelial injury and supports the statement that HBO_2 does not cause inflammation. This result raises new questions regarding the underlying mechanism. Whether HBO_2 can prevent endothelial injury by maintaining the integrity of the glycocalyx and the mechanisms involved should be explored in future studies.

Although HBOT decreases markers of endothelial dysfunction and inflammation, these decreases do not appear to occur through Sirt-1 and eNOS. Sirt-1 is an important protein that can affect the activation of NF-κB and eNOS through the deacetylation process. Sirt-1 also mediates monocyte adhesion and foam cell formation by regulating the expression of VCAM-1 and intercellular adhesion molecule-1 (ICAM-1).[14] Recent studies show that HBO_2 increased Sirt-1 levels, leading to an improvement in endothelial dysfunction.[51,43] The results of this study differ from previous studies, possibly because they provide new information regarding the kinetics of changes in Sirt-1 and eNOS levels. This difference suggests that a certain dose of HBO_2 is needed to increase Sirt-1 and eNOS levels. Thus, further research is needed on the dosage of HBO_2 hormesis that can increase Sirt-1 and eNOS levels.

CONCLUSION

A single HBO_2 treatment did not cause inflammation or endothelial dysfunction, as shown by a decrease in the levels of Syndecan-1, IL-1, and VCAM-1, despite an increase in MDA and NF-κB levels. It is still too early to conclude that Sirt-1 and eNOS are not involved in the

mechanism of preventing inflammation and endothelial dysfunction when administering HBO_2, considering the differences between the doses given and other studies that provide different results. Further research is required to determine the optimal dose of HBO2 hormesis that can increase Sirt-1 and eNOS levels.

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DISCLOSURE

The authors declare that they hold no conflicts of interest regarding this study.

AUTHORS' CONTRIBUTIONS

All authors contributed to the design, conduct, data analysis, and writing of the manuscript. All authors have read and approved the final manuscript.

ETHICAL CONSIDERATION

This research has been ethically approved by the Research Ethics Committee of Universitas Pembangunan Nasional Veteran, Jakarta, Indonesia (25/I/2023/KEPK).

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