

IN VITRO STUDIES ON HEME OXYGENASE-1 AND P24 ANTIGEN HIV-1 LEVEL  
AFTER HYPERBARIC OXYGEN TREATMENT OF HIV-1 INFECTED ON PERIPHERAL BLOOD  
MONONUCLEAR CELLS (PBMCs)

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**Abstract**

**Background:** Heme oxygenase-1 (HO-1) is a protein secreted by immune cells as a part of immune response mechanism. HO-1 can be induced by variety agents that causing oxidative stress, such as exposure to 100% oxygen at 2,4 ATA pressure. It plays a vital role in maintaining cellular homeostasis. This study was conducted to identify the effect of hyperbaric oxygen exposure in cultured of PBMC that infected by HIV-1.

**Material and Methods:** Primary culture of PBMCs were isolated from 16 healthy volunteers and HIV-1 infected MT4 cell line by co-culture. The PBMCs were aliquoted into two wells as control group and treatment group. The 16 samples of HIV-1 infected PBMC were exposed to oxygen at 2,4 ATA in animal hyperbaric chamber for three times in 30 minutes periods with 5 minutes spacing period, that called 1 session.

The Treatment done on 5 sessions within 5 days. 16 samples of HIV-1 infected PBMCs that have no hyperbaric treatment became control group. The supernatant were measured the HO-1 production by ELISA and mRNA expression of HO-1 by real time PCR and the number of antigen p24 HIV-1 by ELISA.

**Results:** The result showed that there was no increasing of HO-1 at both mRNA level and protein level, there was a decreasing number of antigen p24 HIV-1 at the treatment group. In addition, hyperbaric exposure could not increase the expression of HO-1, more over the viral replication might be reduced by other mechanism.

**Conclusions:** Hyperbaric oxygen could increase cellular adaptive response of PBMCs infected HIV-1 through increased expression of proteins that can inhibit HIV viral replication.

**Keywords:** HIV-1, hyperbaric oxygen, heme oxygenase-1 (HO-1), mRNA, qPCR.

**Introduction**

Hyperbaric oxygen (HBO) therapy is exposure of pure oxygen at the pressure more than 1 ATA. During therapy, the haemoglobin reaches 100% saturation and more oxygen is dissolved in the plasma than usual, and high amount of oxygen is delivered to almost all tissues, enhancing blood and tissue oxygenation (Simsek et al., 2011).

Exposure to pure oxygen in HBO therapy with therapeutic doses can increase oxidation products and the effectiveness of antioxidant defence mechanisms to prevent oxidative stress. Increased production of the superoxide radical followed by hydrogen peroxide will impact the transcription factors with their main target protein heme oxygenase-1 (Simsek et al., 2015). Heme oxygenase-1 (HO-1) was increase 24 hours after hyperbaric oxygen exposure suggesting an involvement of HO-1 in the adaptive protection (Speit and Bonzheim, 2003). Heme oxygenase-1 is a protein which is highly inducible by a variety of agents causing oxidative stress and play a vital function in maintaining cellular homeostasis along with increase resistance against oxidant mediated cell injury. HO-1 involved in the adaptive protection against the induction of DNA damage after HBO treatment (Rothfuss et al., 2001).

The previous study reported the role of HO-1 in monocytes against HIV infection, showed that HO-1 suppresses HIV-1 replication in primary human monocytes by PKC-dependent HO-1 induction (Devadas et al., 2010). The other study showed that there was a strong evidence for HO-1 as a novel endogenous biologic component with anti HIV activity (Devadas and Dhawan, 2006). The aim of this study was to identify the effect of HBO whether HO-1 produced after hyperbaric oxygen exposure could inhibit the HIV-1 replication.

## **Materials and Methods**

### **Design Experiment**

This study was approved by the Institutional Ethics Committees of Medical Faculty Airlangga University Surabaya, Indonesia. Informed consent was obtained from all volunteers prior to sample collection. We recruited sample from healthy volunteers purposively with criteria as follows, aged about 18-23 years old, not suffering from chronic illness, not taking drugs such as steroids, cytostatic and drugs and not smoking. Eight milliliters of whole blood were obtained from each 16 healthy volunteers who had approved informed consent. This Peripheral Blood Mononuclear Cells (PBMCs) were further isolated and divided into two groups; treatment group (with HBO) and control group (no HBO).

### **Isolation and Preparation of PHA-stimulated uninfected volunteer Peripheral Blood Mononuclear Cells (PBMCs)**

PBMCs were separated from whole blood freshly collected by using histopaque (SIGMA) density gradient centrifugation prepared from buffy coat. PBMCs were re-suspended in RPMI 1640 (Gibco) medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2mM L-glutamine, 100 µg/ml streptomycin, 10% purified human IL-2 (SIGMA) and 10 µg/ml PHA (SIGMA). The number of viable cells were counted by using trypan blue and hemocytometer. Viable cells look clear, while the necrosis cells (nonviable cells) was blue. PBMCs were aliquoted into two wells of sterile 24 wells plate at concentration  $5 \times 10^5$  cell/µl, one well for control group and one well for treatment group. The uninfected PBMCs were cultured in medium with stimulated phytohemagglutinin (PHA, 10 µg/mL) and incubated for three days in CO<sub>2</sub> incubator. After three days, the culture fluid was incubated in RPMI-1640 medium containing T cell growth factor cytokine IL-2 for three days in CO<sub>2</sub> incubator, to reach the cell concentration of PBMC up to  $1 \times 10^6$  cell/µl.

### **Infection with HIV-1**

Primary PBMCs culture fluid were infected with HIV-1 by co-cultured with HIV-1 MT4 cell line in BSL-3 (Bio Safety Level 3) laboratory, Institute of Tropical Diseases (ITD) Universitas Airlangga, Surabaya, Indonesia. HIV-1-infected PBMCs cultures were experimental units in this study. A total of 32 PBMCs infected cultures consist of 16 samples treatment group (with HBO) and 16 samples control group (non HBO).

### **Exposure to HBO**

The treatment group received HBO exposure. HBO exposure was performed in a hyperbaric animal chamber with temperature controlled (37°C). The experiment were conducted 3 times for 30 minutes, at 2,4 ATA and O<sub>2</sub> 100% and did intermission in 5 minutes with O<sub>2</sub> 21%. HBO exposure was performed at second day post HIV-1 inoculation for 5 days. However the control group did not receive hyperbaric oxygen exposure. After the last HBO exposure, the examination was performed on both treatment group and control group, supernatant that was containing virus were harvested by centrifugation 1500 rpm for 5 min, and were stored at -80 °C for examination. The supernatant was measured for the concentration of HO-1 by ELISA and mRNA expression by qPCR.

### **Quantitative analysis of HIV-1 replication**

The supernatant were assayed for HIV p-24 used ELISA analysis kit (RETROtex, Zeptometrix corporation buffalo, NY) according to the manufacturer instructions. Supernatant were collected to determine viral production level with measurement of HIV-1 p24 antigen. The level of captured p24 was determined by measuring the absorbance in wave length 450 nm using spectrophotometer.

## Quantitative analysis of HO-1 protein and detection of mRNA HO-1 expression by ELISA and real time PCR (qPCR)

The supernatant were assayed for HO-1 using ELISA kit (R&D systems) and the examination level of HO-1 was performed according to the procedures contained on the ELISA kit. To see whether the expression of HO-1 at the gene level is different from the expression at the protein level, we performed the examination by using 2 methods, measured the HO-1 protein by ELISA and measured the expression of the gene that encoding this protein by qPCR.

RNA were extracted from cell culture using the QIA-amp RNA Mini kit (Qiagen, Hilden, Germany). RNA was reversed into cDNA using the SuperScript III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). The ssDNA concentration was determined by using Nano Drop spectrophotometer. While cDNA generation used a reverse transcriptase kit, qPCR was performed using SsoFast™ EvaGreen supermix (Bio Rad) with the primer sets. qPCR reaction was carried in a 10 µl reaction mixture containing 5 µl SsoFast™ EvaGreen supermix (Bio Rad), 1 µl each primer, 2 µl template and 1 µl nuclease free water. HO-1 gene were amplified by PCR using the following primer pairs (sense 5'-CCT GCT CAA CAT CCA GCT C-3', antisense 5'-CTA CAG CAA CTG TCG CCA C-3'). mRNA levels were expressed in relative copy number normalised against GAPDH mRNA. This normalisation against the housekeeping gene is possible if both PCR (HO-1 gene + housekeeping gene) present in the same efficiency.

All data are expressed as the mean value and its standard deviation. Kolmogorov smirnov to analyse the distribution, followed by dependent test to analyse statistic ( $\alpha=0.05$ ).

## Results

### Level Heme Oxygenase-1 (HO-1) after hyperbaric oxygen treatment on PBMCs culture infected by HIV-1

This study was performed on 32 samples of PBMC cell cultures infected with the HIV-1 MT4 virus, which is divided into 16 samples with hyperbaric oxygen treatment and 16 samples as the control group. The number of cells in healthy PBMCs cultured was calculated until  $1 \times 10^6$  cells/µl and the number of HIV-1 MT4 cell line was  $1 \times 10^6$  cells/µl too. The number of cells both in treatment and control group were homogeneous. The result showed that the treatment group concentrations of heme oxygenase-1 protein were slightly higher compared to the control group (Figure 1). Mean on treatment group was 1.38 ng/ml, and on control group was 0.89 µg/ml. Heme oxygenase-1 measurement was conducted 24 hours after the last of the hyperbaric oxygen exposure.

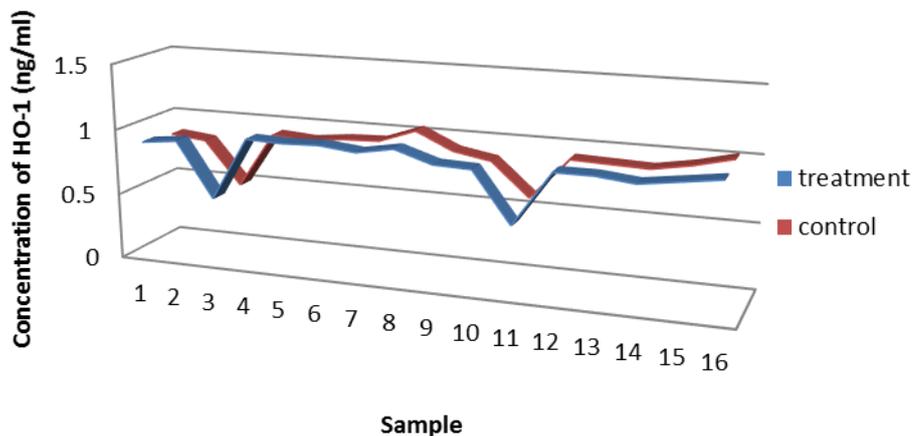
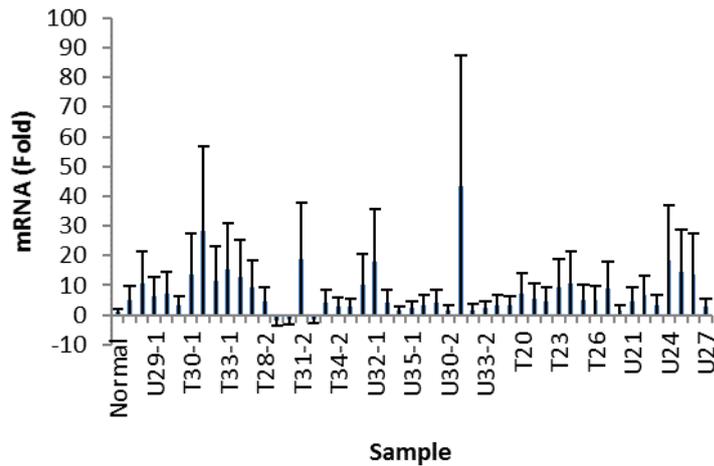


Figure 1: Concentration of HO-1 protein

Expression of Heme oxygenase-1 gene by quantitative RT-PCR obtained the following results as showed in Figure 2.



**Figure 2:** The mRNA expression activity of the gene encoding heme oxygenase-1 after HBO exposure

**Table 1: Difference value between HO-1 and mRNA expression qPCR**

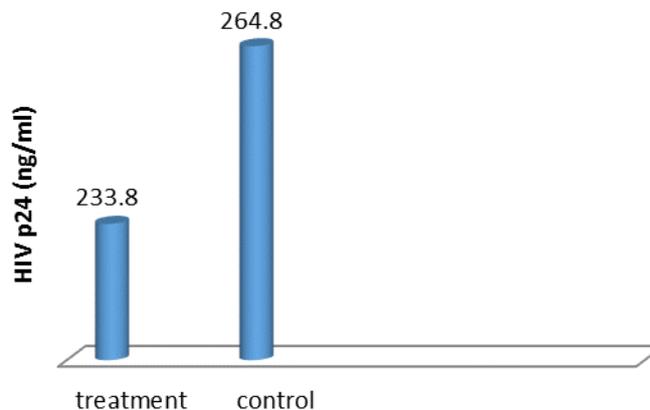
Group of treatment	HO-1 (ng/ml)		HO-1 mRNA (fold)	
	Mean	SD	Mean	SD
HBO Treatment	1.38	2.04	30,980	1,40
No HBO Treatment	0.99	0.15	28,526	1,32
	P= 0,600		P= 0,056	

At the protein level, using the WilcoxonSum Rank test showed a significance level of  $p = 0.600$  ( $p > \alpha$ ), which means there was no difference in the levels of this protein in the treatment and control groups. Although the average levels of HO-1 was higher in the treatment group compared with the control group, there was no difference in protein levels of heme oxygenase-1 after exposure to hyperbaric oxygen between treatment group and control group (Table 1).

At gene expression level, Wilcoxon Sum Rank test results showed  $p = 0.056$  ( $p > \alpha$ ). Means that there were no significant differences in mRNA levels HO-1 between treatment group and control group.

### The number of virus with the HIV-1 p24 antigen

The number of virus was measured by assessing levels of HIV-1 p24 antigen. The results showed the levels of HIV-1 p24 antigen in the treatment group was less than in the control group.



**Figure 3:** The average amount of HIV-1 p24 antigen (ELISA) between treatment and control group

Different test using WilcoxonSum Rank test showed the results of  $p = 0.039$  ( $p < \alpha$ ), which means that there was a significant difference in the amount of p24 antigen levels of HIV-1 between treatment group and control group.

## Discussion

### Analysis of the expression of mRNA and protein levels of the enzyme hemeoxygenase-1

Compared with the control group, the levels of heme oxygenase-1 protein was higher in the treatment group, even statistically there was no significant difference. However, the gene expression of heme oxygenase-1 there were no significant difference expression between treatment group and control group. HO-1 protein is a protein on group of heat shock protein that serve as protective protein in cell mechanisms against stressor. The exposure of HBO on cell will impact on production of free radical that induce the expression of HO-1 gene.

In this study, the level of HO-1 protein was not increase, there was a transcriptional regulatory that affect the expression of protein HO-1 in cell. In samples, with hyperbaric oxygen treatment, sources of oxidants derived from viral replication and hyperbaric oxygen. On the other hand, without hyperbaric oxygen, the oxidant source only derived from viral replication. Treatment control Hemeoxygenase protein, also known as heat shock protein (Hsp) 32, were regulator of transcription factors by cell under stress conditions such as hypoxia, exposure to cytotoxic and hyperthermia (Abdalla et al., 2004), and also by free radical (Gill et al., 2015). The suppression of HO-1 protein levels was identified in cultured macrophages from human brain tissue infected by HIV-1. The decreased in the expression of this enzymes in the macrophage cells was associated with increased oxidative stress due to viral replication and enhancement of glutamate as a result of injury to nerve tissue (Gill et al., 2015). The increase oxidants level in cells due to ongoing viral replication and the presence of reactive molecules of oxygen species (ROS) as a result of exposure to hyperbaric oxygen.

The results showed that this study was not consistent with the proposed research by Devadas and Dhawan, in 2010, that there was existence barriers HIV-1 virus replication by heme oxygenase-1. However HO-1 from their studies did not come from exposure to OHB but was induced by administration of hemin or lipopolysaccharide in the cultured of monocytes. It can be explained that in the treatment group, the levels of ROS is higher than the control group as it gets exposure to ROS derived from HIV-1 viral replication as well as from exposure to OHB, whereas in the control group ROS molecules derived only from virus replication. However, the average levels of heme oxygenase-1 was higher in the treatment group. This suggests that exposure to OHB was able to induce heme oxygenase-1 as a response mechanism that acts as a cell adaptive anti-oxidants than as an antiviral.

### Analysis the number of antigen p24 HIV-1

The result showed that the treatment group had a number of viruses that were significantly lower than the control group. Biomolecular effects of hyperbaric oxygen exposure at therapeutic dose through increasing ROS molecules will activate NF $\kappa$ B as transcription factors which were caused the expression of genes that produce proteins were required in the cell adaptive response. In this condition, HO-1 acts more as an antioxidant than antiviral. The antigen test is a valid measurement to measure productivity of infection. The concentration of HIV-1 p24 antigens in the culture supernatant fluids can be observed after the 5<sup>th</sup> day of viral infection (Yang et al., 2013).

## Conclusion

Exposure with hyperbaric oxygen at 2.4 ATA and 100% oxygen for 3x30 minutes for 5 day in cultured of PBMCs infected HIV-1, was increased the adaptive response of cells by significantly decreased the number of antigen p24 HIV-1, but not in increased the expression of heme oxygenase-1. The decreased number of HIV-1 p24 antigen in the treatment group showed that cell adaptive response result from activation of the transcription factor to produce innate antiviral and inhibit viral replication.

**Conflict of Interest:** Authors declare no conflicts of interest associated with this manuscript. K and N conceived and supervised the study; RB, S and SQK designed experiments; RB and SQK performed experiments; RB and SQK collected samples and clinical information; RB and SQK analysed data; RB wrote the manuscript; RB, K and SQK made manuscript revisions.

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