Hyperbaric hyperoxia exposure in suppressing human immunodeficiency virus replication: An experimental *in vitro* in peripheral mononuclear blood cells culture

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Abstract

Cellular immune has an important role in response HIV infection, which is attack the infected cells to activate signaling molecule. Hyperbaric Oxygen (HBO) worked as complementary treatment for HIV infection. The production of ROS and RNS molecules during hyperbaric exposure can affect gene expression which contributes to cellular adaptative response. This study was conducted to explore the mechanisms of cellular adaptive response to HIV infection during hyperbaric exposure. This study was carried on *in vitro* using healthy volunteers' PBMCs (Peripheral Blood Mononuclear Cells) cultures infected with HIV-1. The study was conducted as a post-test only group design. The experimental unit was PBMC from venous blood of healthy volunteers which were cultured in vitro and infected by co-culturing with HIV-1 in MT4 cell line. The experimental unit consist of treatment and control group. Each group examined the expression of transcription factor NFκB, Interferon α, reverse transcriptase inhibitors (p21), and the amount of HIV-1 p24 antigen. There were increasingly significant differences in the expression of the trancription factor of NFκB, p21, andHIV-1 p24 antigen,as well as mRNA transcription of interferon α2 between treatment and controlgroup. By decreasing p24 antigen showed that HBO exposure was able to suppress HIV-1 replication. The exposure to hyperbaric oxygen at the pressure of 2.4 ATAand 98% oxygen wasable to produce ROS and RNS molecules, which play a role in cellular adaptive responses through increasing the expression of nfκb, p21 and mRNA of interferon α2 plays a role in inhibition mechanism of HIV-1 replication in cells.

Introduction

Human Immunodeficiency virus infection and Acquired Immune Deficiency Syndrome (HIV/AIDS) has infected millions of people worldwide. Almost all areas the world have been infected by HIV infection. Treatment for HIV patients with antiretroviral drugs will suppress the amount of virus in their bodies, prevent transmission, increase life expectancy, and improve quality of life. Since antiretroviral drugs must be consumed everydayfor a lifetime, it often causes psychological effects like depression, as well as other side effects. A 'novel strategy' for a therapeutic method is still needed to eliminate the virus safely from the patients; both therapy or immunological preventive is needed to avoid the usage of antiretroviral drugs.[1] However, such novel strategy has not been identified yet. The main target of HIV is CD4 T lymphocytes which contributes to controlling the immune response during an infection. HIV progression is classified through defective lymphocytes T-CD4, which number and function gradually decreased.[2]An increase in programmed cell death (apoptosis) of lymphocytes T-CD4 was also identified in HIV infection.[3]

The exposure of 100% oxygen with the pressure of 2.5 atmosphere absolute (ATA) in HIV patients can increase CD4+/CD8+ ratio and improve the physical fitness of the HIV patients.[4] Reillo and Altieri[5] showed that the oxygen exposure among 11 HIV patients with T-CD4+ in less than 300 per mm^3 can improve their physical conditions, although it was not known full the molecular mechanisms.[5]

Furthermore, the mechanisms of viral inhibition through the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) modulate the cascade reaction of the transcription factor, growth factor, and cytokines.[6] ROS further increases the expression of NFkB by synthesizing intreferon1 that can activate macrophage and lymphocyte T-CD4+.[7-9] The role of p21 protein in inhibiting HIV viral replication are explored as the impact of oxygen exposure.[10]

The aim of this study is to examine a chain reaction in inhibiting HIV viral replication in PBMCs infected HIV-1 after the exposure of hyperbaric oxygen (HBO) at 2.4 ATA and oxygen 100%.

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Contributions: N and K conceived and supervised the study; RB designed experiments; RB and SQK performed experiments, collected samples and analysed data; RB wrote the manuscript; RB, SQK, N and K revised the manuscript.

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Dedication: The article is dedicated to my institutions and my country to contribute in problem of HIV infections.

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Material and Methods

Study design and inclusion criteria

This study was designed as an experimental study by using HIV-infected Peripheral Mononuclear Blood Cells (PBMCs) cultures and treating it with hyperbaric oxygen. The PBMC from a

healthy volunteer were co-cultured with MT4 cell line-infected HIV. They were divided into two groups, a treatment group with hyperbaric oxygen treatment and control group without hyperbaric oxygen treatment. PBMCs were taken from venous blood of healthy volunteer with criteria as follows: young adults (aged 18-25 years), not in a state of chronic disease (such as tuberculosis, carcinoma, or diabetes mellitus), not consuming long-term immunosuppressive drugs (such as corticosteroids or cytostatics), not smoking, and agreed to sign the informed consent. The subjects were also willing to donate about 8 ml of their blood.

Ethic Statement

This study was approved by the Institutional Ethics Committees of Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia (permission number: 296/EC/KEPK/FKUA/2015).

Co-cultures between HIV-1 infected and uninfected cells

The Human acute T lymphoblastic leukaemia cell lines (MT-4 and MOLT-4) were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma),1% Natrium bicarbonate, 100 U/mL penicillin G, and 100 µg/mL streptomycin (culture medium) and incubated for three days in a CO2 incubator. The Virus persistently infected MT-4 cells (MT-4/HIV-1) which were obtained from a co-culture of HIV-1 infected PBMCs with uninfected PBMCs using stimulated phytohemagglutinin (PHA, 10 µg/mL). The co-culture is incubated in growth RPMI-1640 medium containing T cell growth factor cytokine, IL-2 for three days in CO_2 incubator. After three days of infection, p24 antigen in supernatant was measured by ELISA kit (Zeptometrix co.), according to the manufacturer's instructions.

In vitro HIV-1 replication by treatment of hyperbaric oxygen

The *in vitro* study was performed by treatment of hyperbaric oxygen. This study was evaluated HIV-1 replication**.** One session of treatment consists of 3x30 minutes with 2.4 ATA and 100% oxygen with the interval of 5 minutes (in every 30 minutes exposure) using 20% oxygen. Treatment of hyperbaric oxygen was delivered for five sessions in five days. Examination of the variables performed 24 hours after the last oxygen hyperbaric exposure. The results were compared between treatment group with hyperbaric oxygen treatment and control group without hyperbaric oxygen treatment.

Immunohistochemistry examination of NFκB and p21 protein

Freshly centrifuged cell pellets were used for immunohistochemical analysis. NFκB served as a marker for macrophages and p21 as a marker for determined the inhibition of HIV replication. The cell pellets seeded in gelatin-coated coverslips. The sterilized coverslips were placed in the bottom of 24 wells plate and incubated for seven days in CO_2 incubator. The cell culture was fixed with 10% formaldehyde solution for 20 minutes at room temperature. Thereafter the cells were treated in blocking buffer 45 minutes at room temperature and washed with PBS. The optimal antibody concentration, which gives the best staining with minimum background,using 1:100 dilution. Samples were then incubated for overnight at 2–8 °C with anti-NFκB and p21 as the primary antibody (ratio, 1:100; Abcam). After rinsing in PBS, samples were incubated with biotinylated anti-mouse (ratio, 1:1500; Vector Laboratories). Avidin-biotin peroxidase complexes (Vector Laboratories) were added followed by visualization with 3,3-diaminobenzidine tetrachloride (Vector Laboratories), and washed with PBS. VIP reagent was also added to each well until the desired stain intensity develops through visualizing it using a fluorescence microscope with its filter set with anappropriate label. Finally, the percentage of cells expressing p21 or NFkB protein was calculated among the number of live cells that were found microscopically.

Measurement of Interferon α and HIV-1 p24 antigen

Interferon α and HIV-1 p24 antigen in the supernatants were measured by ELISA kit, according to the manufacturer's instructions (Sigma).

Gene expressions of interferon α2 gene by qPCR

The gene expressions of interferon α2 were quantified by amplification of specific mRNA using qPCR. Total RNA was isolated from culture fluid using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Viral RNA was reversely transcribed into cDNA using the SuperScript III First-Stand Synthesis kit (Invitrogen, Carlsbad, CA) with the reverse primer, K-env-R1, 5'-CCAATCAGGGAAGAAGCCTTG-3' [corresponding to nucleotide 9158 to 9138 of an HIV-1 subtype B reference strain, pNL4-3 (GenBank accession number AF324493)].The purity and concentration of cDNA were measured by nanodrop (Thermoscientific, USA). qPCRreaction was carried in a 10 µL reactionmixture containing 5 µL SsoFast^{TM}EvaGreensupermix (Bio Rad), 1 µL each primer, 2 µltemplate and 1µL nuclease free water. For the amplification of viral interferon of viral forward: 5 'GCA AGT CTG CAA GCT GCT TG 3', and reverse: 3 'GAT GGT CTT TTC AGC TTG GA 5'.The qPCR condition detailed werefor 40 cycles with annealing temperature 58 ^{0}C.For normalizing the gene expression data, we used GADPH as a housekeeping gene. The primers sequence were5 'TTT AAC AGG GCT GCT TCT GGT 3' for forward and 3 'CCC ATT TTG TTG CAC GAG GGA 5' for reverse.

Statistical analysis

All data were analyzed using SPSS 18. The normality test was performed with shapiro wilk to see the distribution of data. If the data were normally distributed should be tested with paired t-test. If the data were not normally distributed should be tested with Wilcoxon. The significance level uses α=0.05. If p<0.05 was considered as significant.

Results

Expression of NFκB

The quantitative enumeration of the cell which expressed NFκB presented that NFkB was significantly different between the treatment group (=0.43%) and the control group (0.20%) (p=0.004, p<α) as showed in Figure 1.

Interferon-α

Interferon-α protein concentration was not significantly different between the treatment and the control group. There was27.45 ng/mL in control group and 28.53 ng/ml in treatment group (p=0.301, p>α)as showed in Figure 2. However, the mRNA expression was significantlydifferent at 22.54-fold in the treatment group and -21.11-fold in control group (p=0.001, p<α) as showed in Figure 3. In the diagram, the normal expression of interferon α2 gene with positive value less than 1 indicates an increase in gene expression more than normal; while a negative value (less than 0) indicates a downstream regulation or decreased expression of the gene. Interferon α-2 is a part of the complex gene of Interferon α.

p21 protein expression (reverse

transcriptase inhibitor)

The p21 protein expression obtained from the percentage of cells expressing p21 protein among the total number of cells which were found in the visual field microscopic examination on immunocytochemistry examination. The number of visual fields were observed in 10 fields of low power microscopic view with a counted average score. Moreover, p21 expression was identified in 0.25% cell in the treatment group, compared to zero in the control group, so it was significantly different (p=0.001, p<α) as shownin Figure 4. For the treatment group, there are cells with blue and brown colours. Blue indicated a viable cell which had no expression p21 protein,and brown indicated a viable cell which hadan expression of p21 protein. However, for the control group, there was only a blue colour found on cells, which is a viable cell that had no expression of p21 protein.

HIV-1 p24 antigen

The quantity of p24 antigen which was examined by ELISA was referred to as the presenting of HIV-1 virus in cell. The result of p24 was 233.8 ng/ml in the treatment group and 264.8 ng/mL in control group, which obviously statistically different (p=0.039, p<α; α=0,05). It showed that HBO exposure was significantly protective against the HIV virus.

Discussion

The study was started by developing infected PBMC of a healthy donor through co-culturing with MT4 cell line infected with the HIV-1 virus. It was known that HIV-1 virus was able to multiply continuously in MT4 cell line and produce infective HIV-1 viruses, due to its high sensitivity and permissive effects for the virus.[12-13] PBMCs cell infection was mediated by CXCR4 receptor in T lymphocyte cell.[14] The $1x10^6$ cell/mL density was successfully targeted to be infected by HIV-1 virus *in vitro*.

The increasing of NFκB was substantially different in an exposed group from non-exposure HBO (p=0.00, p<α). The oxidative stress, which is caused by HIV infection or HBO, also induces the expression a number of genes that regulated by transcription factor, such as NFκB.[15] This phenomenon was also found in the previous study that hyperbaric oxygen administration of 2.4 ATA during 3x30 minimum 5 sessions increased inducible nitric oxide synthase (iNOS) and NFκB expression.[16] They were also found to notably fasten wound healing in the treatment group. NFκB is also able to regulate apoptotic cell, which contributes to a role in the immune system against infection.

Interferon α protein slightly increased in the expression of protein level, but it was statistically different in the mRNA transcription level, which interferon α2 mRNA concentration was substantially higher in the treatment group rather than the control group. It showed that HBO

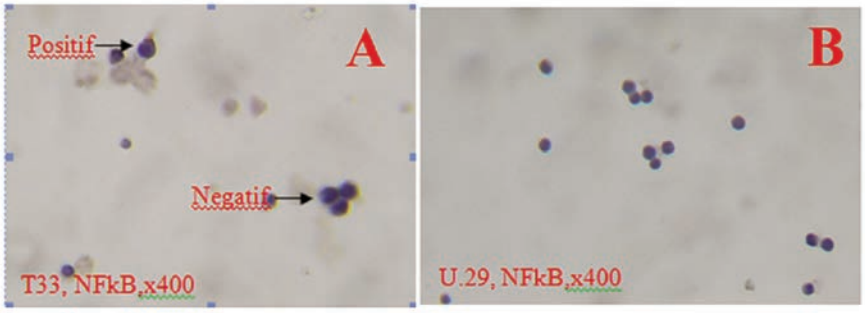

Figure 1. Immunocytochemical features from HBO treataed samples, blue and brown cell images are living cells that express the NFκB protein (a) and control samples showing live blue colored cells that do not express the NFκB protein (no brown image appears) (b).

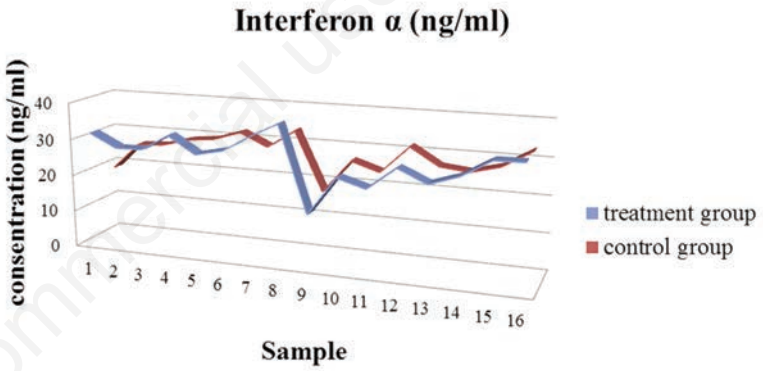

Figure 2. Interferon α concentration after hyperbaric treatment by ELISA.

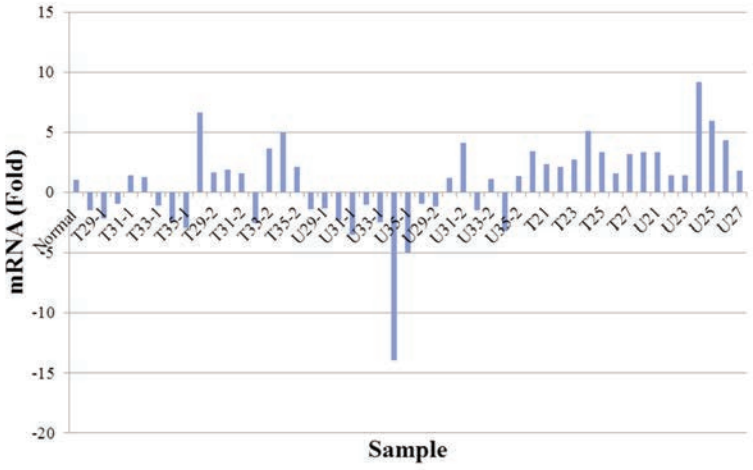

Figure 3. Gene expression of Interferon α 2 after hyperbaric treatment (U= untreatment, T= treatment) by realtime PCR.

exposure induced the expression of interferon α, but in the *in vitro* environment, the protein translation process was completely hard as in *in vitro* environment. Interferon α is the interferon type 1 which can be induced in the primary culture of *in vitro* cells infected with the virus.[8]

It is widely known that gene encoding ofinterferon α consists of 13 genes which present in the short arm of chromosome no. 9; thus dividing the α interferon into 12 subtypes, *i.e.* interferon α1,α2,α4,α5,α6,α7,α8,α10,α14,α16,α17 and α21.[8,17,18] In this study, only mRNA α-2 was measured as an indicator for the expression of interferon α. However, The expressions of all interferon α genes including mRNA α-2 were derived from the activation of the same transcription factor, such as IRF (Interferon Regulating Factor) 3 and IRF-7.[17] In addition to activating the same transcription factor, the interferon α encoded by different genes that has the same receptors to run in the cell. They are called the common receptor Interferon α receptor 1 (IFNAR 1) and interferon α receptor 2 (IFNAR 2).[17, 9,20] The interferon α is encoded by a relatively large structural gene, the expression in which the subtype arises from interferon α depends on the type of cell, as well as the type of stimulation given.[21]

The previous study of hyperbaric oxygen exposure showed that after exposure to HBO 1 ATA 100% O_2, a qPCR examination was performed to see the expression of HO-1 and Hsp 70 genes using beta-actin as a housekeeping gene.[22] There was a 2.1-fold increase of HO-1 expression relative to control, while Hsp 70 gene expression increased 5-folds at 2.4 ATA exposure.[22]HO-1 didn't increase after HBO exposure by *in vitro*. If HO-1 didn't increase after HBO exposure, no antioxidants were involved in response to the increase of reactive oxygen system (ROS) molecules. It also illustrated that the exposure of HBO (2-4 ATA and oxygen 98%) on cultured monocyte cells and human macrophages can induce cytokines at mRNA level and protein level in the first 12 hours.[23]

Analysis of p21 expression

This study proved that there was a significant increase in protein expression p21 in the treatment group compared to the control group.Protein p21 (Cip1/Waf1) is a cyclin dependent kinase inhibitor (CDKI) which acts as a regulator in cell cycle in phase G1 and phase Son mitosis.[24] Hyperbaric oxygen exposure can induce H_2O_2 molecules as ROS molecules which can increase the expression of p21 protein through activation of mitogen activated proteinkinase (MAPK). There is a cross-talk between the redox state within the cell and the signaling pathway inside the cell, where an elevated ROS molecule triggers cellular responses, such as cell cycle cessation, apoptosis or necrosis, depending on the degree of the damage.[25-26]

In the event of HIV infection, protein p21 contributes to the inhibition of HIV-1 virus replication in macrophages and CD4 T lymphocytes. In some HIV-infected patients, this protein is naturally present and capable of suppressing viral replication.[27] This mechanism also occurred through activation of NFκB transcription factor.[10] This study showed that the increase of protein p21 also identified an increase of transcription factor NFκB.

Nitric Oxide (NO) is also generated in hyperbaric oxygen delivery, where the NO molecule also has a role in the regulation of p21 expression through the extracellular signal regulated kinase (ERK) pathway.[28]

The increasing of p21 was followed by decreasing p24 HIV-1 in the treatment group which means that the replication of HIV virus was hampered. It is suggested that p21 may inhibit HIV viral replication by blocking the reverse transcriptase enzyme, by blocking molecules in the host cell responsible for dNTP supply (as a reverse transcriptase enzyme), and by blocking the necessary CDKI2 enzymes for the effectiveness of the reverse transcriptase enzyme in CD4 lymphocyte cells.[10,29]

It showed that in the treatment group, the average number of cells is higher than the group without treatment, but not statistically significant. This is because apoptotic cell death in this study was not only influenced by elevated levels of p21, but also by other factors, such as HIV viral replication. Additionally, p21 protein serves to regulate the cell cycle and contributes to the inhibition of apoptosis by binding with procaspase 3, caspase 8, and apoptosis signal regulating kinase (ASK).[30]

Analysis of p24 HIV-1 antigen

The effects of hyperbaric oxygen exposure through an increased ROS molecule interchangeably influence the activation of the NFκB transcription factor which directly induces the production of necessary proteins in cellular adaptive responses, such as antioxidants as natural antivirals. The antiviral proteins in this study were interferon α and protein p21, which only protein p21 prove to be correlated closely with a number of viruses in this study. The concentration of HIV-1 p24 antigen in culture supernatant fluid was observed after day five of viral infection.[30]

A decrease in the number of HIV-1 p24 antigens in the treatment group reveals that cellular adaptive responses resulting from the increased activation of the NFκB. While a transcription factor was directed at the viral replication barrier through increasing p21 as a reverse transcriptase inhibitor.

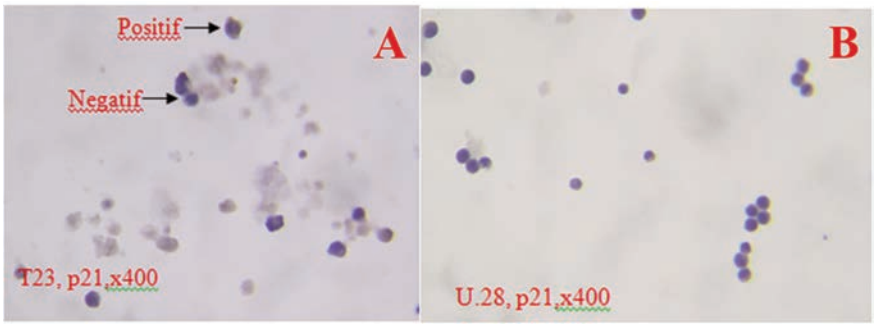

Figure 4. p21 Expressions by Immunohistochemistry from (a) treatment group and (b) control group.

Conclusions

Hyperbaric oxygen exposure to HIV-1-infected cells led to several signaling processes in the cell, such as stimulating NFκb, interferon α, and p21, all of which affect each other to reach a decrease in the number of antigen p24 HIV-1 or inhibiting HIV virus replication. This study was conducted *in vitro* cell cultures, so the immune system involved in response to the increase of ROS molecules within the culture cell is not as complete as *in vivo* process. However, it is possible that the protein expressed by the induction of transcription factors becomes less optimal, compared to experimental animals or humans. The new findings revealed that the hyperbaric oxygen delivery at a pressure of 2.4 ATA, O2 100%, 3x30 min/session, and

for five sessions significantly decreased the amount of p24 HIV-1 antigen through the increased expression mechanism of NFκB and protein p21 which are reverse transcriptase inhibitor in HIV-1 virus replication process. There was also an increase in the expression of interferon α2 gene as indicated by the increase of its mRNA.

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