RESEARCH ARTICLE

Hyperbaric Oxygen Ameliorates The Expression of Tumor Growth Factor-β and Malondialdehyde in Pristane-induced Lupus Nephritis Mice Model

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Abstract

BACKGROUND: Lupus nephritis (LN) is associated with chronic renal failure and a high mortality rate. Tumor growth factor-β (TGF-β) plays a basic part in keeping up immune homeostasis, meanwhile extensive oxidation of the lipid membrane in LN causes the arrangement of malondialdehyde (MDA). Up to date, the mechanism of hyperbaric oxygen (HBO) therapy in LN has not been elucidated well. Hence this study was conducted to assess the impact of HBO therapy on TGF-β and MDA expression in the pristane-induced LN mice model.

METHODS: Thirty-two mice aged 8-12 old weeks were isolated into 4 groups: normal saline-injected mice (group 1); pristane-injected mice (group 2); as well as pristane-injected followed by HBO-exposured with 2.4 (group 3) and 1.5 (group 4) atmosphere absolute (ATA) pressure. Pristane were injected intraperitoneal to initiate LN. Two months after pristane injection, the mice were placed within 24 hours in the special cage for metabolic examination to determined that pristane-induced LN mice model was successfully formed and marked by the occurring proteinuria. Oxidative stress was assessed by examining MDA, while systemic inflammation was assessed by examining by TGF-β. MDA and TGF-β serum were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS: A significantly lower (p<0.050) MDA expression was found in group 3 in comparison with group 2. The results also showed that TGF-β level was significantly increased (p<0.050) in group 3 compared to group 2.

CONCLUSION: HBO therapy ameliorates inflammation in LN by diminishing MDA and expanding TGF-β levels through activating antioxidants.

KEYWORDS: lupus nephritis, hyperbaric oxygen, malondialdehyde, tumor growth factor-β

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Introduction

More than half of systemic lupus erythematosus (SLE) patients progress with lupus nephritis (LN) (1), which might be dangerous manifestation of the organ.(2) LN increments in morbidity, end-stage renal disease, and mortality.(3) About 60% of SLE become LN and 30% result in end-stage kidney failure.(4)

Up to date, there are no curative medications for lupus. (2) The current standard of therapy include a combination of corticosteroids and cyclophosphamide or mycophenolate mofetil (MMF).(5,6) Significant side impacts occur on patients with renal damage related to this treatment.(6)

LN is caused by a primary immune disease and also due to impaired redox homeostasis.(7) The increase in oxidative stress is associated with LN.(8) Kidney medulla has lower oxygenation because of the fewer blood supply

and more prominent oxygen utilization.(9,10) LN is known to be related to oxidative stress due to persistent and uncontrolled inflammation.(10,11) Kidney tends to be hypoxic in LN since immune complex deposition.(9)

The main regulator of immune homeostasis is transforming growth factor-β (TGF-β), which is a multifunctional cytokine that can either inhibit or stimulate cellular proliferation.(12,13) The roles of TGF-β in the early autoimmune and late organ damage phases of LN are paradoxical.(14) A downregulation of TGF-β in an inflammatory context had been proven.(15,16) Total TGF-β levels were lower in LN patients with high disease activity and severe organ damage.(16,17)

TGF-β is required to suppress the activation of B cell and plays a part in the induction of immune tolerance.(18) TGF-β is an anti-inflammatory cytokine that plays a role in healing tissue injury in LN.(17) In LN patients, there was a failure in apoptotic cell clearance that leads to the decrease in production TGF-β.(19) Study about lupus-prone F1 mice showed a decreased of TGF-β in lymphoid tissues, but the TGF-β, mRNA, TGF-β receptor type II, and phosphorylated Smad3 in the kidneys and urine were increasing in the correlation with local tissue fibrosis. TGF-β levels, measured by Western blot, were decreased in the spleen, but increased in the kidneys. Cultured spleen cells also decreased the TGF-β1 expression.(14)

The TGF-β serum was found to be reduced in LN, but urinary TGF-β levels were significantly increased in LN patients. There was no significant correlation between serum TGF-β1 levels and 24 hours urine protein.(17,20). TGF-β in LN decreased because T regulatory cells (Tregs) in active LN were lower compare to the control. Tregs maintains self-tolerance by suppressing the auto-reactive lymphocytes through secreting TGF-β as anti-inflammatory cytokines. (21) So any therapy for LN involving TGF-β depends on the function of when and where the cytokine acts.(16)

Extensive oxidation of the lipid membrane in LN causes the formation of lipid-derived reactive aldehydes (LDRA), along with malondialdehyde (MDA).(8) MDA serum level suggest oxidative stress level in whole the body.(22) Elevated MDA levels are associated with tissue damage in LN.(23)

Hyperbaric oxygen (HBO) therapy is a treatment in which a patient is exposed to 100% oxygen (O_2), at a pressure higher than atmospheric pressure.(24,25) It increases the oxidative capacity of the blood serum by diffusing it.(24) HBO therapy corrects hypoxia and reduces tissue edema. (26) This treatment increments the expression of diverse growth factors.(27) The use of HBO therapy shows a hormetic effect by inducing reactive oxygen species (ROS) at moderate levels.(28) Moderate concentrations of ROS are useful for cell signaling and the immune system.(23) Previous research has proven that HBO therapy increased TGF-β.(29) This therapy has also been shown to reduce MDA levels.(30)

The current study aimed to develop an adjuvant treatment along with standard therapeutic to decrease side effects of LN standard therapy. HBO therapy as an adjunctive therapy for LN has not been widely studied. Therefore, this study was conducted to determine whether HBO ameliorates inflammation in LN by diminishing MDA and expanding TGF-β levels. Since LN model mice with pristane injection have been commonly used, and known to be the best model to explore LN (31-33), hence the same model was used for this study.

Methods

LN Animal Model

Female BALB/c mice aged 8 weeks and weighed 25±3 gram were obtained from Biosains Laboratory, Universitas Brawijaya University, Indonesia. All mice were feed with adequate sterile food and water, and kept in the cage with light and dim for 12:12 hours. The temperature in the room was maintained at 22±1°C, within a positive laminar flow. Each cage contained a maximum of 8 mice. Before the study, all mice were acclimatized for 7 days at the Biosains Laboratory, Brawijaya University.

Previous study with a single intraperitoneal injection of pristane in BALB/c mice aged 2 months, showed that an LN animal model had been formed which was characterized by significant proteinuria compared to the control mice at 2 months after pristane injection.(33) Another study also showed similar results.(32) Proteinuria is the main clinical manifestation of LN with a prevalence of 100%. (3) The pristane-induced LN mice model in this study were formed from female BALB/c mice that were given a single injection of 0.5 cc or 0.783 g/mL pristane intraperitoneally (Santa Cruz Biotechnology, Inc. SC-281684).(33) The urine examination of mice was carried out on day 60.(11) Administration of pristane injection indicated that the result of LN model mice characterized by proteinuria (+3 or ≥ 500 mg/dL).(3,11)

Animal Treatment

Simple random sampling was used to divide all mice into four groups (n=8, each group): group 1 received

intraperitoneal injection of a single dose 0.5 mL normal saline as placebo and did not treated with HBO therapy; group 2 received a single injection of 0.5 cc or 0.783 g/mL pristane intraperitoneally and did not treated with HBO therapy; group 3 received a single injection of 0.5 cc or 0.783 g/mL pristane intraperitoneally and exposed to HBO with 2.4 atmosphere absolute (ATA) pressure; while group 4 received a single injection of 0.5 cc or 0.783 g/mL pristane intraperitoneally and exposed to HBO with 1.5 ATA pressure.

This study was obtained a permission from the Ethics Committee of Medical Faculty, Airlangga University, Surabaya, Indonesia (No. 17/EC/KEPK/FKUA/2022). The animal care, experimentation, and disposal followed standard protocols. The damage to mice was kept to a minimum.

Administration of HBO Therapy

Group 3 and 4 were admitted for HBO at the Animal Research Facility of Naval Health Institute, Surabaya, Indonesia. Group 3 were gotten HBO treatment with 100% oxygen beneath 2.4 ATA in the animal chamber. Each treatment session comprised breathing the air in the pressure steadily come to 2.4 ATA within 10 minutes. Then continued by breathing oxygen 100% within 90 minutes. The breathing of oxygen was split into three sections and two intervals of breathing the air within 5 minutes. The length of each session was 30 minutes. After that, breathing the air in pressure steadily diminished to normal (1 ATA) within 10 minutes. Group 3 got treated for HBO therapy for 10 sequential days. This HBO therapeutic dose was in accordance with the United States Navy Treatment Table 9 to improve tissue healing and avoid toxic doses of oxygen. Group 4 received almost the similar treatment as group 3, the only difference was instead of given 2.4 ATA pressure, 1.5 ATA pressure was used.

Proteinuria Measurement

The urine examination of mice was carried out on day 60 after pristane injection and 9 days since the first HBO therapy. The mice were placed for 24 hours in a metabolic cage. The urine protein samples were analyzed using the reagent strips Medi-Test Combi10®SGL (Macherey-Nagel GmbH & Co.KG, Düren, Germany).

Enzyme-linked Immunosorbent Assay (ELISA) Analysis

Thirty minutes after group 3 and 4 finished receiving the 10 days HBO treatment, all samples were anesthetized utilizing ketamine intraperitoneally. Blood samples were taken from the ventricles of the mice's heart. The levels of serum MDA measurement were carried out using Malondialdehyde ELISA Kit from The Bioassay Technology Laboratory system (Catalogue No. E0625Mo, Korain Biotech, Shanghai, China). Serum was obtained after centrifugation of blood samples at 2000-3000 RPM for 20 minutes. Fifty µL of standards were added to the standard well, while 40 µL of samples and anti-MDA antibodies were added to sample wells. Fifty µL of streptavidin-HRP was added to sample wells and standard wells, then mixed. The plate was covered with a sealer before incubated for 60 minutes at 37°C, and washed 5 times. Fifty µL of substrate solution A followed by 50 µL substrate solution B were added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark. After 50 µL stop solution was added to each well, the color was developed. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after. The concentration of MDA serum was plotted on a standard curve to calculate sample concentration.

The levels of serum TGF-β measurement were carried out using a Tumor growth factor-β ELISA Kit from The Bioassay Technology Laboratory system (Catalogue No. E0285Mo, Korain Biotech) according to the manufacturer's instruction for use, similar to the previously serum MDA measurement. Anti-double stranded DNA (anti-dsDNA) antibodies serum as a predictive marker of disease activity was also examined with ELISA.

Statistical Analysis

The IBM Statistical Product and Service Solutions (SPSS) version 24.0 (IBM Corporation, Armonk, NY, USA) was used to analyze the data statistically. All descriptive data were described by mean and standard deviation. The statistically significant difference results were indicated by $p<0.05$.

Results

Pristane-induced LN Mice

The urine examination of mice showed that group 1 which received normal saline injection had negative results, while group 2, 3 and 4 which received pristane injection had positive results (+3 or ≥ 500 mg/dL). There was no significant difference in proteinuria occurrence after and before HBO therapy ($p=0.3170$), as shown in Figure 1.

The result of the ELISA test also indicated that the anti-dsDNA antibodies serum in group 3 was lower than in

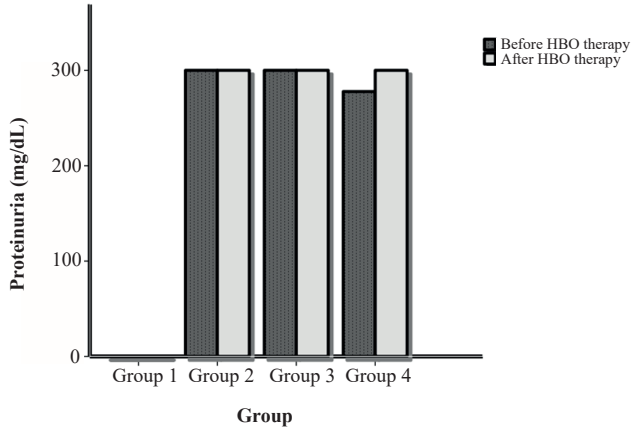

Figure 1. Urine protein concentration between groups before and after HBO therapy. Each bar showed the mean urine protein concentration (mg/dL) which was measured using a dipstick. The urine protein concentration of all mice was monitored at 60 days of pristane injection and 9 days following HBO therapy.

group 2 with significant value of p=0.011, however there was no significant difference between group 4 and group 2 (p=0.491).

HBO Therapy Effects on MDA Level in LN Mice

One-Sample Kolmogorov-Smirnov test for normality test indicated that the data of serum MDA were normally distributed, and the Lavene test of serum MDA showed homogeneity in variation.

The analysis of variance (ANOVA) and the least significant difference (LSD) test of serum MDA showed that group 1 and group 3 was significantly lower than group 2 (p=0.000 and p=0.020, respectively). But there was no significant difference between group 2 as compared with group 4 (p=0.179) (Table 1).

HBO Therapy Effects on TGF-β in LN Mice

The One-Sample Kolmogorov-Smirnov test for normality test indicated that the data of serum TGF-β had normal distribution, and the Lavene test of the TGF-β showed homogeneity in variation.

ANOVA and LSD test of serum TGF-β represented that the mean level of TGF-β from group 1 as compared with group 2 showed no significant difference (p=0.088). Group 3 was significantly higher than group 2 (p=0.000), while group 4 that also received HBO therapy but with different doses showed no significant difference compared to group 2 (p=0.958) (Table 2). The description of comparative test analysis of MDA and TGF-β levels between groups were represented in Figure 2.

Discussion

LN is a major cause of morbidity and mortality as well as the most severe complications of SLE. The significant side impacts in patients with renal damage are related to a high dose of immunosuppressive drugs for this treatment. (6) Hence, new therapies still need to be discovered. In this research, the LN animal model can be made by exposing BALB/c mice to pristane. It can induce the LN model similar to LN condition in humans.(11,34) LN is marked by proteinuria.(33) Proteinuria in the pristane-induce LN mice had been made after 2 months of pristane injection. According to classification International Society of Nephrology/ Renal Pathology Society (ISN/RPS), LN Class I or II has a normal renal function, microhematuria, and proteinuria (proteinuria >3 g/day), 2 months after pristane injection is the condition consideres as the early stage of LN.(15) The result of the study also showed that anti-dsDNA antibodies increased after 2 month pristane injection, but decrease after being given HBO therapy with 2.4 ATA following 10 days. Anti-dsDNA antibodies as the predictability markers have sensitivities of 80% and specificities of 90%.(35)

HBO therapy with 2.4 ATA and 1.5 ATA following 10 days did not affect proteinuria. According to the previous study about the correlation between serum TGF-β and disease-related variables in LN patients, the result showed that variable serum TGF-β in LN patients with 24 hour urinary proteins did not significantly differ.(17)

Administration of pristane injection increased levels of MDA in animal models. The mean MDA levels of the LN model that received HBO in group 3 decreased and were

Table 1. Description of MDA between experimental groups.

Group	MDA (nmol/mL)			
	Mean±SD	Min.	Max.	*p*- value*
Group 1	0.24±0.04	0.15	0.28	0.000
Group 2	0.37±0.07	0.29	0.48	
Group 3	0.30±0.05	0.23	0.35	0.020
Group 4	0.33±0.34	0.29	0.39	0.179

*Mean difference was compared to group 2, significant if p<0.05.

Table 2. Description of TGF-β between experimental groups.

Group	TGF-β (ng/L)			
	Mean±SD	Min.	Max.	*p*- value*
Group 1	79.33±2.16	77.06	84.05	0.088
Group 2	74.99±3.42	70.08	79.60	
Group 3	87.09±7.35	78.33	101.94	0.000
Group 4	75.11±2.88	71.05	79.21	0.958

*Mean difference was compared to group 2, significant if $p<0.05$.

significantly different when compared to the LN model that did not receive HBO in group 2. These results are in accordance with a study conducted on Wistar albino rats given HBO therapy 2.4 ATA which showed an increase in MDA levels on the first day, but the levels decreased after the 10th day.(36) This current study also used a pressure of 2.4 ATA for 10 days, but in contrast to the previous study conducted that used Wistar rats and the duration of each session was 60 minutes, this study used Mus musculus BALB/c mice and a duration of 90 minutes instead.

Research with similar results was also reported in a study examining patients with chronic wounds who were given HBO therapy 2.2 ATA for 60 minutes each session. The outcome showed a decrease which was significant in MDA between the last session of day 20 and day one.(27) Although using a lower HBO pressure and shorter time per session, as well as a longer duration of therapy, the results are the same as this study which showed that HBO therapy could reduce MDA levels.

The mean MDA level in the group receiving HBO therapy was still higher than the group 1 (0.24±0.04) nmol/mL receiving normal saline injection although not significantly different. This is probably because in this study HBO therapy was only carried out for 10 days or 10 sessions.

MDA levels are markers of oxidative stress.(37) Oxidative stress is caused by the peroxidation of fat cell membranes can observed by examining the level of MDA.(8,38) LN is associated with oxidative stress.(12) Expanded peroxidation of cell membrane lipid in LN can cause the arrangement of lipid-derived reactive aldehydes (LDRA) including MDA.(8,26) Oxidative stress affects the inflammatory process in LN. MDA levels can decrease with an increase in antioxidant enzymes.(27) HBO therapy can increase ROS but its function is to activate antioxidant enzymes by protecting lipid membranes from excess peroxidation.(26)

The mean TGF-β level in LN model mice that received HBO therapy in group 3 increased and was significantly different when compared to the LN model that did not receive HBO therapy in group 2. The administration of HBO therapy increases TGF-β which means that the increase in TGF-β indicates improvement of LN. The mean TGF-β showed a decrease after the mice received pristane

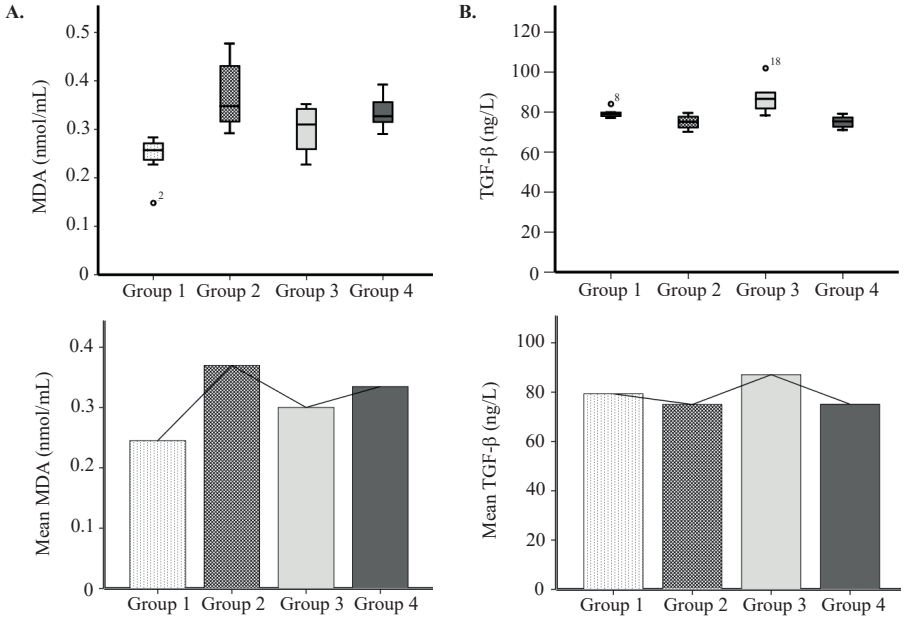

Figure 2. Comparative test results of MDA and TGF-β levels between groups. Each bar showed the mean±SD, data were analyzed using one-way ANOVA. A: Mean of serum MDA level; B: Mean of serum TGF-β level.

injection, but there was no significant difference between group 1 (79.33±2.16) ng/L injected with normal saline and group 2 (74.99±3.42) ng/ L who received pristane injection. This outcome similizes to another *in vivo* study using a glioma model given HBO 3 ATA for 1 hour. The study also showed that HBO therapy can increase TGF-β levels. (29) Although the study used a higher pressure and shorter duration of administration than this study, it was still within safe pressure limits.

Research that supports the results of this study was also proven previously that report a result on HBO therapy for traumatic brain injury using rats with a pressure of 2 ATA for 1 hour given daily for 14 days showed that HBO therapy increased TGF-β as anti-inflammatory.(39) The function of TGF-β as a growth factor. Inhibitory cytokines, such as TGF-β, play an important role in maintaining tolerance and immune homeostasis. The anti-inflammatory cytokine TGF-β is required to suppress B cell activation induced by toll-like receptor (TLR) stimulation.(12) Inflammation can also be ameliorated by TGF-β through inhibition of the transcription factor nuclear factor-κB (NFκB).(40)

Lupus patients experience a loss of phagocytic function and cell viability distinct from marginal zone macrophages (MZM). This leads to failure of clearance of apoptotic cells and decreased production of TGF-β.(17) So that TGF-β levels decrease in LN patients. Research on HBO therapy is still very limited, especially regarding the effect of HBO therapy on LN through changes in TGF-β levels. Existing research on HBO on changes in TGF-β levels that have been carried out showed that TGF-β levels increased after HBO therapy in Glioma.(29)

HBO therapy in this study increased TGF-β in animal models. Inflammation from LN is expected to decrease with elevated TGF-β levels. The level of TGF-β after pristane injection and normal saline injection decreased but did not statistically different probably because the role of TGF-β in maintaining tolerance and immune homeostasis did not work alone, but was also influenced by other cytokines such as interleukin (IL)-10. In addition, it may take a long time after pristane injection until there is a significant decrease in TGF-β mice. This study shows that HBO therapy can reduce MDA levels so that it can reduce the degree of activity of animal models of LN.

Conclusion

This study shows that HBO therapy with 100% oxygen beneath 2.4 ATA within 90 minutes, splitting into three sections and two intervals of breathing the air within 5 minutes can ameliorate LN by reducing disease activity with decreasing the levels of MDA and TGF-β serum in early stage LN in the pristane-induced LN mice model. The effectiveness of HBO therapy is determined by the HBO therapeutic doses, which are the pressure used and the length of exposure. Appropriate therapeutic doses can produce beneficial effects. The outcomes of this study indicated that HBO therapy can decrease LN activity by lowering levels of MDA and increasing TGF-β. HBO therapy is expected to be an alternative therapy to reduce morbidity and mortality in LN patients.

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Authors Contribution

S and TH were involved in planning and supervised the work. IDM and TH performed the measurements, processed the experimental data, performed the calculations and statistical analysis, drafted the manuscript and designed the figures. S, IDM and TH aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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