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Hyperbaric oxygen as an immunosuppressant in mouse model of lupus nephritis

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ABSTRACT

Hyperbaric oxygen (HBO) therapy has been used for many conditions. The immunosuppressive effect of HBO is also reported. Thus, HBO is thought to be used for the treatment of lupus nephritis (LN). The current research intended to determine the effect of HBO 2.0 ATA by inhaling ±100% oxygen (O_2) 3 times for 30 min, for 10 consecutive days as an immunosuppressant in an animal model of LN. A total of 21 BALB/c female healthy and active mice (body weight 25-30 g and 8-12 weeks old) were divided into 3 groups including the group of control mice (G1), the group of LN mice without HBO therapy (G2), and the group of LN mice with HBO therapy (G3). The results showed a significant reduction of anti-dsDNA Ab and proteinuria levels in the HBO (G3) compared to the LN group not given HBO (G2). Repair of kidney tissue damage was characterized by a decrease in inflammatory cells, a decrease in tubular constriction and a decrease in immune complex deposits in the group given HBO (G3) compared to the group not given HBO (G2). HBO can reduce anti-dsDNA Ab levels and proteinuria, and repair damaged kidney tissue. In conclusion, the findings suggest the HBO as an immunosuppressive in LN in mice.

INTRODUCTION

Lupus nephritis (LN) is an autoimmune disease as one of the clinical manifestations of systemic lupus erythematosus (SLE) which is the main cause of morbidity and death due to the most severe complications of the kidney. Generally, immunosuppressive drugs are used to control this disease, but currently available immunosuppressive drugs do not provide optimal results [1, 2]. The incidence of lupus varies between countries [3]. The average prevalence is around 50 in 100,000 people [4] and the female-male ratio is 7-15:1 (adults) and 3-5:1 (children). Female dominance results from a complex interaction between sex hormones, genetics, epigenetics, and gut microbiota. LN is among the top 20 main causes of death for women aged 5-64 years [5]. Mortality is highest in African, Asian, Hispanic, and Native American populations [4].

In SLE, there is dysregulation of various immunological pathways, production of autoantibodies, and activation of the complement system, ultimately causing a loss of autoimmune tolerance. LN is characterized by proteinuria, the formation of anti-nuclear antibodies, and inflammation [1]. Anti-dsDNA antibodies (anti-dsDNA Ab) have a sensitivity of 80% and a specificity of 90% for diagnosing LN [6]. Anti-dsDNA Ab are specific biomarkers for LN that contribute to damage to organs including the kidneys [2, 7]. Inflammation causes the kidneys to become more hypoxic during LN and the prognosis becomes worse if renal hypoxia occurs [8].

Hyperbaric oxygen (HBO) is a kind of therapy using breathing media with oxygen levels of 100% or higher than normal air in a room with air pressure higher than 1 absolute atmosphere (ATA). The principle of HBO therapy is to increase the partial pressure of oxygen to the body's tissues [9]. This method has beneficial effects related to increasing microcirculation and facilitating the diffusion of oxygen from the capillaries to the tissues, with an increase in the partial pressure of oxygen reducing hypoxia and tissue edema [10]. HBO was initially used as primary therapy in diving cases. In its development, HBO has been widely used in the treatment of many clinical cases, but side effects have hampered and limited the clinical application and promotion of HBO therapy.

A previous study stated that HBO is more likely to cause adverse reactions or side effects if the room pressure is above 2.0 ATA and the treatment is given for more than 10 consecutive sessions [11]. These side effects include ear discomfort, sinus pain, eye side effects, seizures, claustrophobia, chest pain, gastrointestinal reactions, headache, fatigue, and congestive heart failure [11]. Therefore, the right dose of HBO is needed to get optimal results with minimal side effects. The correct dose of HBO therapy according to the hormesis theory is needed for clinical improvement in LN patients. Thus, the effect of HBO therapy at a dose of 2.0 ATA by inhaling ±100% oxygen (O_2) 3 times for 30 minutes for 10 consecutive days on anti-dsDNA Ab, proteinuria, and histopathological changes in kidney tissue in animal models of LN needs to be studied.

MATERIALS AND METHODS

Ethical approval

This research was conducted after obtaining a certificate of ethical merit from the Naval Health Institute Research Ethics Commission No. 08/EC/LKS/V/2023 (Research period May 2023 – August 2023).

Animals and housing conditions

Twenty-one *Mus musculus* BALB/c female mice with 25-30 g body weight and 8-12 weeks old were used. Experimental animals were placed in cages in the laboratory in the form of plastic boxes (40 cm long x 30 cm wide x 18 cm high). The base of the cage was given a material that can absorb water, namely wood shavings (Figure 1A). The room temperature was around 24-26^{o}C with humidity 45-55%. Then the animal adaptation was carried out for 7 days.

Experimental approach

Preliminary research was conducted on 5 female *Mus musculus* BALB/c mice which were injected with a single dose of pristane at 0.5 cc intraperitoneally to induce LN. Anti-dsDNA Ab levels before injection and after observation for 1 month were assessed statistically. The sample size in the study was calculated based on the results of preliminary research calculated using the formula from Charan J and Biswas T in 2013 as follows [12]. Accordingly, the minimum sample size for each group of r' or replication is 7 mice. 21 experimental mice were used for 3 experimental groups such as G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group. G2 and G3 were given intraperitoneal injections of pristane, a single dose of 0.5 cc to induce LN (Figure 1B). Observations were made for 1 month.

On the 31st day, the characteristics of mice suffering from LN were examined. Apart from anti-dsDNA Ab examination, proteinuria examination was also carried out using URS-10T reagent strips for urinalysis. The results were accounted significant when the value was ≥ +3 (≥ 3.0 g/l).

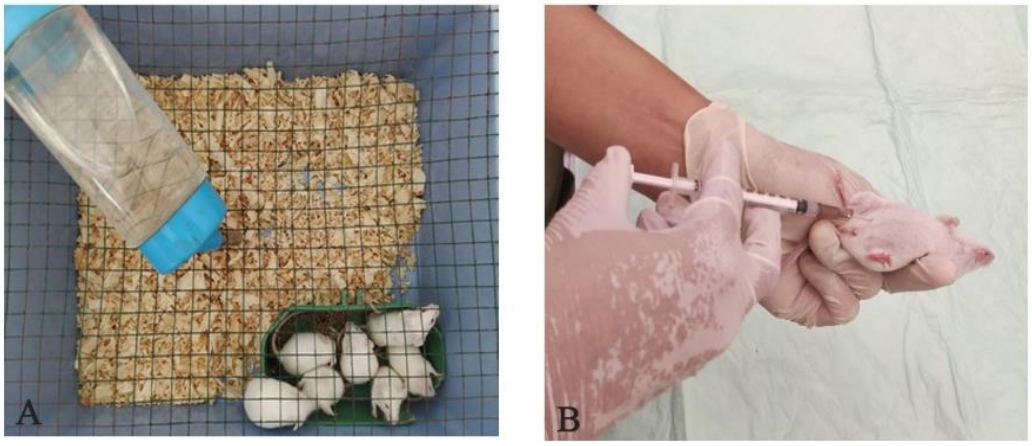

Figure 1. A) Group of experimental mice in the cages. B) Mice were injected with Pristane intra-peritoneally.

Procedures of HBO therapy

On the 32nd day, group 3 was exposed to HBO equipped with a manometer to see the depth of pressure levels of O_2 gas and CO_2 gas in the room. Also, a humidity meter to measure air humidity and a thermometer to measure the temperature in the animal chamber were used (Figure 2).

HBO therapy was carried out in the morning at 08.00 West Indonesian Time (WIB) for 10 sessions for 10 consecutive days at the same time. The experimental animal model of the LN (G3) group was treated with HBO to 2.0 ATA or 10 meters, inhaling normal air or 21% O_2 levels for 15 minutes, then breathing with 98-99% O_2 for 90 minutes divided by 3 each 30 minutes with interspersed 2 times 5 minutes breathing with normal air. After that, 10 minutes of inhaling normal air while reducing the pressure until it returns to normal pressure or 1 ATA. During therapy, the gas analyzer showed oxygen levels of 23.5%, carbon dioxide levels of 0.01%, air humidity of 50%, and temperature of 28°C in the hyperbaric animal chamber.

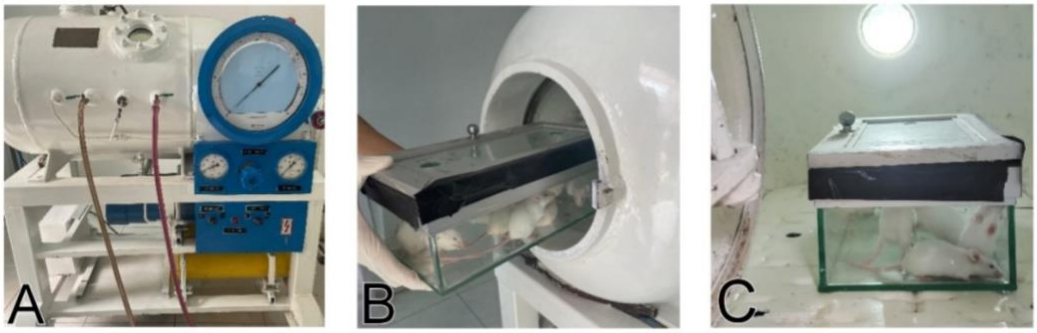

Figure 2. A) Hyperbaric animal chamber. B) The process of inserting experimental treatment group animal models into the hyperbaric animal chamber. C) The treatment group animal model received oxygen therapy in a hyperbaric animal chamber.

Sample collection

On day 42nd, sampling was carried out 30 minutes after the treatment group (G3) finished HBO therapy. Experimental animals in all groups including G1, G2, and G3 were anesthetized with an intraperitoneal injection of ketamine at 50-100 mg per kilogram of body weight plus xylazine 10 mg per kilogram of body weight. It was confirmed that the mice had been anesthetized with signs of no pain response. Blood sampling was performed after the mice were anesthetized for 10 min. The blood was taken using a syringe in a peripheral vein through the tail and heart ventricles. Then euthanasia was performed using a dose of ketamine 4-5 times the anesthetic dose. Then the kidney tissue of mice was taken for histopathological examination. Dead mice were buried.

Analysis of anti-dsDNA Ab

On day 43, the collected blood samples were placed in a vacutainer containing ethylene diamine tetra acid (EDTA). The Ab anti-dsDNA level was examined with the Mouse Anti-double Stranded DNA ELISA Kit Bioassay Technology Laboratory (Korain Biotech Co., Ltd, Shanghai, China) (standard curve range 0.5 ng/ml-200 ng/ml, sensitivity 0.25 ng/ml, size 48 well) and read by ELISA reader (ng/ml). Briefly, all reagents were prepared and placed at room temperature before use. Then the number of strips is determined and inserted into the appropriate place for testing, and 50µL of the standard is added to the standard well. 40µL of sample was added to the sample well then anti-Ab anti-dsDNA was added to the sample well, then 50µL of streptavidin-HRP was added to the sample well and standard well. Then mixed thoroughly and the plate was covered with a sealer and incubated for 60 minutes at 37°C. The sealer was removed, and the plates were washed 5 times with 0.35 ml of wash buffer for 30 seconds to 1 minute for each wash. For automatic washing, all wells were aspirated and washed 5 times with wash buffer. 50µL of substrate solution A was added to each well, then 50µL of substrate solution B was added to each well. The plate was incubated and covered with a new sealer for 10 minutes at 37°C in a dark place. Then, 50µL stop solution was added to each well, and then the blue color was immediately changed to yellow. An ELISA reader (Sumifin Citra Abadi., Ltd, Tangerang, Indonesia) was used and set to 450 nm within 30 minutes after adding the stop solution. The optical density (OD value) was determined.

Analysis of proteinuria

Urine collection was carried out by placing the mice in a cage containing urine for 1 day. The urine collected in the holding container was taken using a 1 cc disposable syringe and then put the urine into a tube for laboratory examination. Proteinuria was examined with URS-10T reagent strips (Zhejiang Orient gene Biotech Co., Ltd, Huzhou, China) for urinalysis with a sensitivity of 0.15-0.3 g/L.

Histopathological analysis of kidney

After each animal was sacrificed, the kidneys were carefully dissected for histopathological examination. Kidney tissue samples were taken and then fixed and stored in 10% neutral buffer formalin (NBF) for 48 hours. After that, the samples were washed with 10% phosphate buffer solution for 3 hours and dehydrated using a series of ascending alcohol levels (70%, 80%, 90%, 95%, 100% each for two hours, and finally

100% overnight). After dehydration, samples were cleaned with xylene and embedded in paraffin. The paraffin-embedded blocks were cut into sections 4–6 μm thick using a rotary microtome near a 56°C water bath, for location development of network pieces. After expanding, the tissue is captured with a glass object, dried at room temperature, then put in the incubator before coloring. The samples were soaked in xylene I, II, and III solutions for 5 minutes each, dehydrated with ethanol solutions I and II for 5 minutes each, and washed with distilled water for 1 minute. Then, soaked in Hematoxylin solution for 15 minutes, rinsed with running water, washed with Lithium carbonate for 15-30 seconds, rinsed with distilled water for 1 minute, and dipped in acid alcohol for 4 dips. Then samples were rinsed with distilled water for 1 minute and 15 minutes. After that, the samples were stained with Eosin for 4 minutes. The samples were placed in 70%, 80%, and 96% alcohol for 3 minutes each. Next, the samples were soaked in ethanol solutions III and IV for 3 minutes each, and then in xylene solutions IV and V for 3 minutes each. Then, the preparation was dried and dripped with paramount adhesive and covered with a cover glass. The images were captured using a light microscope (Olympus IMS, Tokyo, Japan) at 400x magnification (200μm).

Statistical analysis

All data were analyzed statistically using IBM SPSS version 22.0. The data were considered significant if the p-value < 0.05. The measurement results were tabulated to be displayed descriptively.

RESULTS

Effect of HBO on anti-dsDNA Ab levels in LN mice

The normality test using the Shapiro-Wilk test had a significance of $p > 0.05$, and this showed that the data were normally distributed in all groups. The homogeneity test using the Lavene test had a significance of $p < 0.05$, indicating that there was a variant or the data was not homogeneous. The Kruskal Wallis test showed a statistically significant ($p < 0.05$) difference from the mean anti-dsDNA Ab levels of the three groups including G1, G2, and G3 (Table 1).

In the G3 group, the average anti-dsDNA Ab value (42.18429 ± 5.746552 ng/mL) was lower than that of the G2 group (62.07571 ± 4.731919 ng/mL). The Mann-Whitney test also showed that anti-dsDNA Ab levels in the G3 group were significantly ($p<0.05$) decreased compared to the G2 group (Figure 3).

Table 1. Differences in the results of anti-dsDNA Ab in experimental groups.

Anti-dsDNA Ab levels (ng/mL)			
Group	**Mean ± SD**	**SEM**	**p**
G1	17.40286 ± 1.994122	0.753707	
G2	62.07571 ± 4.731919	1.788497	0.000
G3	42.18429 ± 5.746552	2.171993	

Data are shown as mean ± SD (standard deviations); SEM (Standard error of the mean); $p<0.05$ was considered statistically significant; G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group.

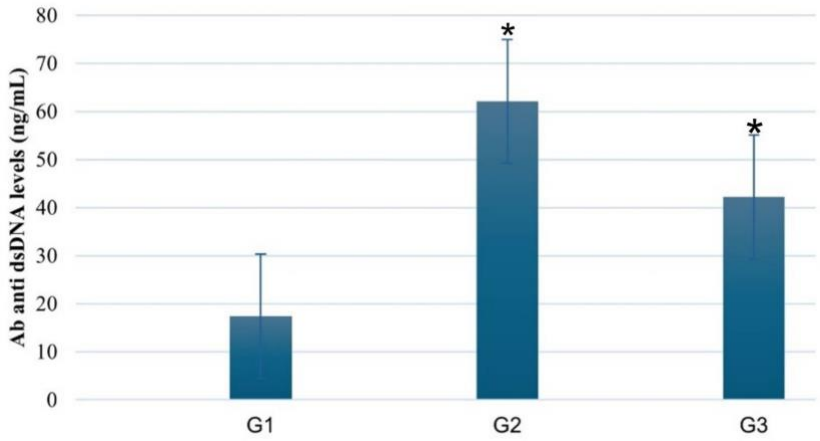

Figure 3. Diagram of differences in mean anti-dsDNA Ab levels in groups G1, G2, and G3. The average value of ab anti-dsDNA levels in those who received HBO (G3) decreased significantly compared to those who did not receive HBO (G2). G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group. * Indicates a significant difference ($p<0.05$), *on G2 vs G1, and * on G3 vs G2.

Effect of HBO on proteinuria in LN mice

The normality test using the Shapiro-Wilk test had a significance of $p < 0.05$, and this showed that the data were normally distributed in all groups. The homogeneity test using the Lavene test had a significance of $p < 0.05$, indicating that there was a variant or the data was not homogeneous. The Kruskal Wallis test showed a statistically significant ($p < 0.05$) difference from the mean proteinuria levels of the three groups including G1, G2 and G3 (Table 2).

In the G3 group, the average proteinuria value (1.57143 ± 0.975900 g/L) was lower than that of the G2 group (10.28571 ± 9.086882 g/L). The Mann-Whitney test showed that proteinuria levels in the G3 group were significantly ($p<0.05$) decreased compared to the G2 group (Figure 4).

Table 2. Differences in the results of proteinuria in experimental groups.

Proteinuria (g/L)			
Group	**Mean ± SD**	**SEM**	**p**
G1	0.15000 ± 0.000000	0.000000	
G2	10.28571 ± 9.086882	3.434519	0.000
G3	1.57143 ± 0.975900	0.368856	

Data are shown as mean ± SD (standard deviations); SEM (Standard error of the mean); $p<0.05$ was considered statistically significant; G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group.

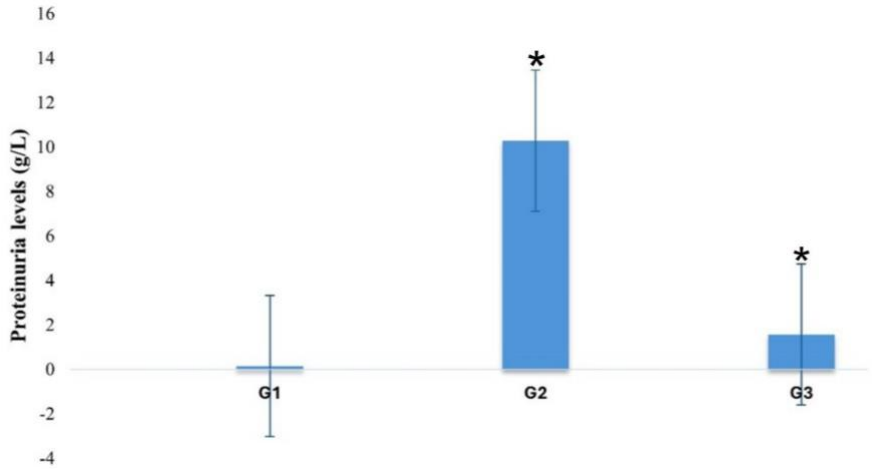

Figure 4. Diagram of differences in mean proteinuria levels in groups G1, G2, and G3. The average value of proteinuria levels in those who received HBO (G3) decreased significantly compared to those who did not receive HBO (G2). G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group. * Indicates a significant difference ($p<0.05$), *on G2 vs G1, and * on G3 vs G2.

Effect of HBO on kidney damage in LN mice

The results of the histopathological analysis with the HE staining technique showed that there was no kidney damage in the G1 group, whereas, in the G2 group, there was kidney damage as characterized by inflammatory cells appearing swollen with an enlarged cytoplasmic volume, narrowed tubules, and deposits of immune complexes. In G3 with HBO therapy, there was repair of kidney damage which was marked by a decrease in inflammatory cells, a reduction in tubular constriction, and a decrease in immune complex deposits. Below are the results of histopathological changes in the 3 research groups including G1, G2, and G3 (Figure 5).

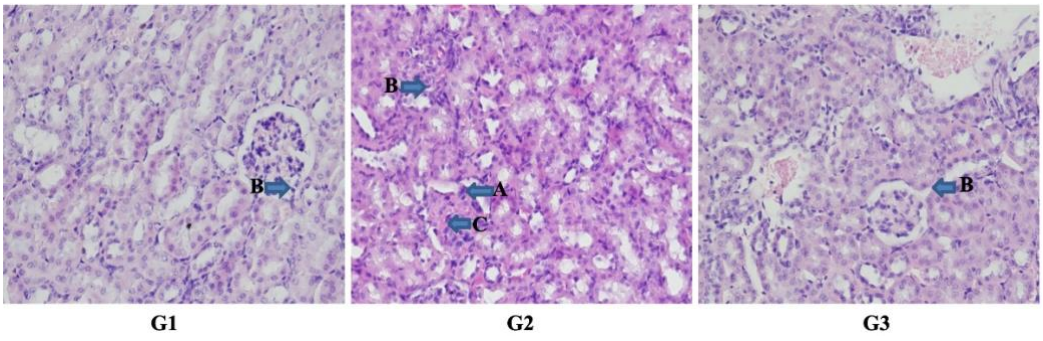

Figure 5. Expression of changes in kidney tissue in groups G1, G2, and G3 by a histochemical technique using a light microscope, shown at 400x magnification (200μm). A: Inflammatory cells appear swollen with an enlarged cytoplasmic volume. B: constricted tubules. C: deposits of immune complexes. In the group given HBO therapy (G3), there was an improvement in kidney damage which was characterized by a decrease in inflammatory cells, a decrease in tubular constriction, and a decrease in immune complex deposits compared to the group not given HBO therapy (G2). G1= control group; G2 = LN + non-HBO group; and G3 = LN + HBO group.

DISCUSSION

The kidney itself is a unique organ which physiologically more hypoxic than any other organ, with an oxygen gradient of about 50 mm Hg (6–7%, compared to 21% in air) in the cortex and less than 10 mm Hg (less than 1%) in the corticomedullary junction. Physiologically, the need for oxygen is not as big as the heart and lungs. The hypoxic

environment of the kidney for too long a time determines T-cell function, which causes subsequent tissue damage [13]. Thus, a condition called hormesis is needed by an organ or cell of living things according to their respective needs.

The concept of hyperbaric oxygen dose is derived from the definition of HBO as a drug. HBO does include O_2 level, pressure depth, duration, interval, and frequency. This study used a pressure of 2.0 ATA which was lower than the previous study with a pressure of 2.4 ATA [14]. The pressure used was lower than previous studies because it was expected that with a lower oxygen pressure (pO_2), the risk of forming reactive oxygen species (ROS) would be lower, as stated by a previous study [15]. The study stated that high pO_2 caused ROS [15]. Lower ROS levels are expected to stimulate an increase in antioxidant activity resulting in a decrease in oxidative stress which can reduce inflammation [16,17]. Research by Kim et al demonstrated a protective effect of ROS against diseases mediated by the immune system. HBO therapy can increase cellular level ROS in hyperoxic tissues, thereby modulating immune system-mediated disease by enhancing Treg function, which plays an important role as an immunoregulator [18].

During HBO therapy, the oxygen pressure in the arteries is around 1500 mmHg, and in the tissues, 200-400 mmHg. A diffusion gradient occurs from the capillaries to the mitochondria and there is an increase in the amount of dissolved oxygen in the plasma. Oxygen diffuses strongly through cell membranes to all body tissues experiencing hypoxia, including the kidneys. Effective metabolism is highly dependent on the length and duration of the therapy interval [19,20]. HBO therapy overcomes hypoxia resulting in a decrease in the expression of hypoxia-inducible factor-1α (HIF-1α). HIF-1α is influencing and regulating transcription factor expression of the genes involved in maintaining homeostasis such as changes in oxygen concentration. The mechanism of HBO hyperoxia causes decreased HIF1α activity. Decreased HIF1α expression activates FOXP3 gene expression and ultimately degrades RORγt in Th17/Treg progenitors causing polarization from Th17 to Treg. Decreased Th17 and increased Tregs led to a significant reduction in pro-inflammatory cytokine production. This results in a direct decrease in autoreactive B cell proliferation, differentiation, and plasma cell activity [21].

Immune complexes consist of autoantibodies and activate self-dsDNA which are intracellular DNA sensors in immune cells. Autoantibodies are produced by autoreactive B cells assisted by autoreactive T cells. Anti-dsDNA Ab is responded to by pDC through DNA sensors, thus increasing the production of IFN-1 to stimulate dendritic cell differentiation and maturation to activate T cells and induce antibody production by B cells which affect the pathogenesis of SLE [22]. There was a significant decrease in anti-dsDNA Ab levels in the LN model group which was given HBO compared to the LN model animals which were not given HBO (Figure 3). Anti-dsDNA Ab is a subtype of antinuclear antibodies that target antigens in dsDNA [4]. Anti-dsDNA antibodies have been included as a diagnostic criterion in the American College of Rheumatology (ACR) and Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) European Alliance of Associations for Rheumatology (EULAR) classification are now the most recent in 2019. Anti-dsDNA Ab binds chromatin fragments in the mesangial matrix (early stage of the lesion) and on the glomerular basement membrane (late stage of nephritis). Anti-dsDNA Ab in mice binds to the glomerular basement membrane and causes deposition of chromatin complexes with immunoglobulins and can bind to non-nucleosome proteins (annexin A2) which are involved in inflammatory pathways [23].

The pathogenesis of SLE is also characterized by impaired clearance of apoptotic and necrotic cells, resulting in the release of autoantigens. Anti-dsDNA can bind to DNA antigens or cross-reactive antigens in kidney cells, resulting in activation of the complement cascade with immune cell infiltration and cytokine release. This process causes kidney inflammation and fibrosis [24]. Failure to clear apoptotic cells leads to secondary accumulation of necrotic cells. Extracellular high mobility group box 1 (HMGB1) is released from damaged cells so that it can function as a damage-associated molecular (DAMP) template that activates the immune system and causes inflammation by binding to receptors for advanced glycation end products (RAGE), such as receptor 2 (TLR2) and TLR4 [25,26]. TLRs are a group of glycoproteins that function as surface or trans-endoplasmic membrane receptors involved in innate and adaptive immune system responses, transmit widely in immune cells such as neutrophils, monocytes (macrophages), lymphocytes, and dendritic cells (DC) and their activation causes an immune response that can recognize DAMP, extracellular HMGB1 interacts with RAGE, TLR2, and TLR4 on the cell membrane to activate core factor-κB (NF-κB), activate TLR2 and TLR4 through the primary differentiation response of myeloid 88 (Myd88) in a ROS production-dependent manner, resulting in increased secretion of proinflammatory cytokines and chemokines [27].

There was an improvement in kidney tissue damage in the LN group which was given HBO compared to the LN-only group which was not given HBO. This could be due to decreased levels of anti-dsDNA Ab, because it can cause a decrease in inflammation and immune complex deposits. This study following previous research stated that HBO increases levels of oxygen radicals which can stimulate antioxidants, but if the dose is excessive, it can cause oxidative stress. The anti-inflammatory action of HBO is demonstrated by decreasing the concentration of several pro-inflammatory markers. Furthermore, HBO can stimulate the release of angiogenesis-promoting cytokines, including growth factors. In previous studies reporting the relationship between oxidative stress and pro-inflammation, it was remarkable that HBO led to a more anti-inflammatory state [28].

In this study, it was also found that there was a significant reduction in proteinuria levels in the LN group treated with HBO compared to the LN group. Proteinuria is a marker of kidney tissue damage. The pathogenesis of proteinuria is related to inflammation, anti-dsDNA Ab, and the location of immune complex deposits in the kidneys [29,30]. HBO therapy can induce changes in the cluster of differentiation 4 (CD4)/CD8 ratio and reduce lymphocyte proliferation. In addition, it can reduce the spontaneous production of immunoglobulin, proteinuria, Ab anti-dsDNA, and immune complex deposits. HBO therapy produces oxygen free radicals and ROS, but repeated exposure increases antioxidant enzymes such as SOD, glutathione peroxidase, and catalase activity, all of which are enzymes that protect cells from oxidative stress [31]. Repeated HBO exposure can induce important elements of angiogenesis such as expression of vascular endothelial growth factor (VEGF) and endothelial progenitor cells (EPCs). The ratio of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide hydrogen (NAD^+/NADH) increases through the Krebs cycle and mitochondrial metabolism. Sirtuin 1 (SIRT1) increases which functions to deacetylate the forkhead box O_3 ($FOXO_3a$), induces an antioxidant response through modulation of superoxide dismutase 2 (SOD_2) and catalase (CAT) [18].

Lupus disease does not start as a primary immune disease but involves disrupted redox homeostasis resulting in increased apoptosis, impaired phagocytes, and autoimmunity. So that the HBO dose in this study is expected to cause hormesis referring to the adaptive response of biological systems to a moderate environment to increase functionality and

tolerance to more severe disturbances. This research proved that administering 100% HBO 2.0 ATA O_2 therapy with a duration of 3x30 minutes, with 5-minute intervals of breathing normal air for 10 consecutive days had proven to have a positive effect on reducing anti-dsDNA Ab and proteinuria in experimental LN mice model. HBO could also repair damaged kidney tissue by reducing inflammation, reducing tubular narrowing, and reducing immune complex deposits in experimental animals.

CONCLUSION

In conclusion, HBO therapy as an immunosuppressive can reduce levels of anti-ds DNA Ab in blood serum, levels of protein in urine, and repair damage to kidney tissue in mouse model of LN. Thus, HBO therapy is expected to be used as adjuvant therapy along with standard drug therapy in preventing the progression of LN in order to improve clinical symptoms.

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AUTHOR CONTRIBUTIONS

TH: Conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, writing - original draft, writing - review & editing. RY: Formal analysis, investigation & methodology. HEA: Formal analysis, methodology & visualization. LPW: visualization & writing – review. LKA: writing – review & editing. All authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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