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Early hyperbaric oxygen therapy attenuates disease severity in lupus-prone autoimmune (NZB × NZW) F1 mice

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Abstract

The effects of hyperbaric oxygen (HBO_2) therapy on the immune system are reported including potential changes to the CD4/CD8 ratio and a decreased proliferation of lymphocytes during exposure. The immunosuppressive effect of HBO_2 had been suggested to be applicable for the treatment of certain autoimmune diseases. (NZB × NZW) F1 hybrid mice, the unique lupus-prone mice, have been used for elucidating the pathogenesis of SLE. To investigate the effect of HBO_2 on NZB/W F1 lupus-prone mice, 32 female mice were divided into four groups. Three groups of mice were treated with HBO_2 (2.5 atm abs (ATA) for 90 min daily over 2 weeks) starting at (A) 3 months, (B) 6 months, or (C) 8 months of age, while the remaining group (D) served as control. Animals were followed until 11 months of age. Experimental parameters included life span, proteinuria, peripheral lymphocytes, anti-dsDNA antibody titers, and renal histopathology. HBO_2 treatment resulted in increased survival, decreased proteinuria, alterations in lymphocyte-subset redistribution, reduced anti-dsDNA antibody titers, and amelioration of immune-complex deposition in groups A and B. Our data demonstrated that HBO_2 therapy attenuated disease severity in NZB/W F1 mice. HBO_2 treatment may be of use in the clinical treatment of lupus patients and would benefit from further study.

Keywords: Hyperbaric oxygen; Autoimmune disease; Systemic lupus erythematosus; New Zealand mice

Introduction

Hyperbaric oxygen (HBO_2) therapy has been applied as an adjuvant treatment in carbon monoxide poisoning, decompression sickness, air embolism, refractory osteomyelitis, and burn injuries [1–3]. HBO_2 increases blood oxygen concentration, blood flow to ischemic areas, and flexibility of red cells and reduces tissue edema and gas bubble size, although disadvantages of this treatment include barotrauma, neurotoxicity, and other sequelae [4,5]. Oxygen is essential for proper cellular repair of tissue damage in certain injuries, such as burns and crush injuries, and oxygen is utilized by immune cells to kill bacteria, viruses, and parasites [6,7]. Although HBO_2 therapy increases tissue oxygen concentrations, its ultimate effects on the immune system are still unclear.

Warren et al. first demonstrated the immunosuppressive effect of hyperbaric oxygen [8]. Exposure of experimental animals to HBO_2 induced the suppression of several cell-mediated immune responses, such as allograft rejection and delayed type hypersensitivity responses to tuberculin protein [9,10]. HBO_2 exposure also produced changes in lymphocyte redistribution, suppression of antibody production, and suppression of leukocyte and macrophage function [11,12]. Recent evidence suggested that hyperbaric oxygen might modulate cytokine action and prevent cognitive dys-

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function, leading to a reexamination of its therapeutic potential in immunological disorders [13,14].

HBO_2 therapy suppressed experimental allergic encephalomyelitis in myelin-induced guinea pigs [8,15] and prevented the development of arthritis in rats [16]. In other autoimmune disease models, such as NZB and MRL/lpr mice, HBO_2 treatment attenuated spontaneous immunoglobulin production, proteinuria, facial erythema, and lymphadenopathy [17].

Among the murine lupus models, (NZB × NZW) F1 (NZB/W F1) mice are the most similar to the human lupus phenotype, including female dominance, proteinuria, autoantibody production (especially anti-dsDNA antibody), and glomerulonephritis [18]. The present study investigated the effect of HBO_2 therapy on various phenotypic parameters of NZB/W F1 mice. Further, the differential effects of temporal changes in HBO_2 therapy were assessed.

Materials and methods

Mice

Thirty-two female NZB/W F1 mice (1-month old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred under specific pathogen-free conditions at the Animal Resource Services Facility located at the National Defense Medical Center. Mice were treated with HBO_2 (2.5 atm abs (ATA) for 90 min daily over 2 weeks) according to the following group designations: group A (HBO_2 at 3, 6, and 8 months of age); group B (HBO_2 at 6 and 8 months of age); group C (HBO_2 at 8 months of age); and group D (control group, no treatment).

Hyperbaric oxygen treatment

Mice caged in groups of six to eight were exposed to HBO_2 in a hyperbaric animal chamber (0.5 ATA/min to a pressure of 2.5 ATA) for 90 min once a day. The chamber was maintained at 95% oxygen, and CO_2 was exhausted at 10–12 L/min. At the end of the treatment, the chamber was slowly decompressed (0.5 ATA/min).

Experimental protocol

Mice were monitored daily and received a full examination at 2-month intervals. Urine was obtained for measurement of protein, and blood was obtained via the retro-orbital venous plexus for assessment of anti-DNA antibodies, total IgG/M, and FACS analysis. Moribund mice were terminated and necropsied, and kidney sections were prepared for histopathological examination.

Proteinuria

Urine samples were obtained every 2 weeks for protein measurements by Labstix (Bayer Corp., USA). A corresponding grade of 0 to 4 was given to urine protein concentrations with 0 to 30, 30 to 100, 100 to 300, 300 to 2000, and over 2000 mg/dl, respectively.

Flow cytometry

Single cell suspensions of peripheral blood mononuclear cells (PBMC) and splenocytes (depletion of erythrocytes by AEC lysis buffer) were prepared in PBS containing 0.1% (w/v) BSA and 0.1% (w/v) NaN_3 (PBS–BSA–NaN_3). Aliquots were individually stained by anti-CD3–FITC, anti-CD19–PE, anti-CD4–PE, and anti-CD8–PE monoclonal antibodies (mAbs) and analyzed on a FACScan (Becton–Dickinson, Richmond, CA, USA) using forward and side scatter to gate out dead cells and debris, as previously described [19].

Autoantibody determination

Sera for anti-DNA analysis were collected from whole blood in nonheparinized tubes. IgG antibodies to dsDNA were assayed as previously described [19]. Briefly, calf thymus DNA (Sigma Chemical Co., USA) was reconstituted in 0.5 mg/ml in PBS and frozen in aliquots at −20°C. DNA-coated plates were prepared, and microtiter plates were coated with 0.1 ml of 10 μg/ml methylated BSA (Sigma Chemical Co.) in PBS and left overnight at 4°C. Plates were washed three times with PBS before the addition of 0.05 ml of 2.5 μg/ml dsDNA and incubation overnight at 4°C. Next, plates were washed three times with PBS including 0.05% Tween 20 (PBS/Tween) and blocked with 0.1 ml of 1 mg/ml gelatin–PBS for 2 h at room temperature or overnight at 4°C. Serum was diluted 1:50 (previously determined to be the optimal dilution) in gelatin–PBS and added to each well in 0.05-ml aliquots. The standard curve was made by serial dilution of known concentrations of anti-DNA B cell hybridoma soup (generously donated by Dr. B-L. Chiang, National Taiwan University, Taipei, Taiwan), and EU was determined by optimal concentration. Following incubation for 1 to 1.5 h at room temperature, three washes with PBS/Tween were performed. Horseradish-peroxidase-conjugated goat anti-IgM or IgG (Jackson ImmunoResearch Laboratories, Inc., USA) was added to gelatin–PBS at a 1:5000 dilution and incubated for 1 h at room temperature. After three washes with PBS/Tween, the reaction was initiated with 50 ml of 0.55 mg/ml 2.2′-axino-bis-3-ethylbenthiazoline-6-sulfonic acid in citrate buffer (pH 4.2) including 0.03% (v/v) H_2O_2 and terminated by the addition of 0.05 ml of 5% (w/v) sodium dodecyl sulfate (SDS). Each sample was tested in duplicate. Background levels were represented by gelatin–PBS and known positive and negative sera were used as controls. Results were read at 420 nm using a SPECTRAmax reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Serum IgG/M concentration

Serum IgG/M concentrations were analyzed by ELISA, as previously described [20]. Microtiter plates were coated

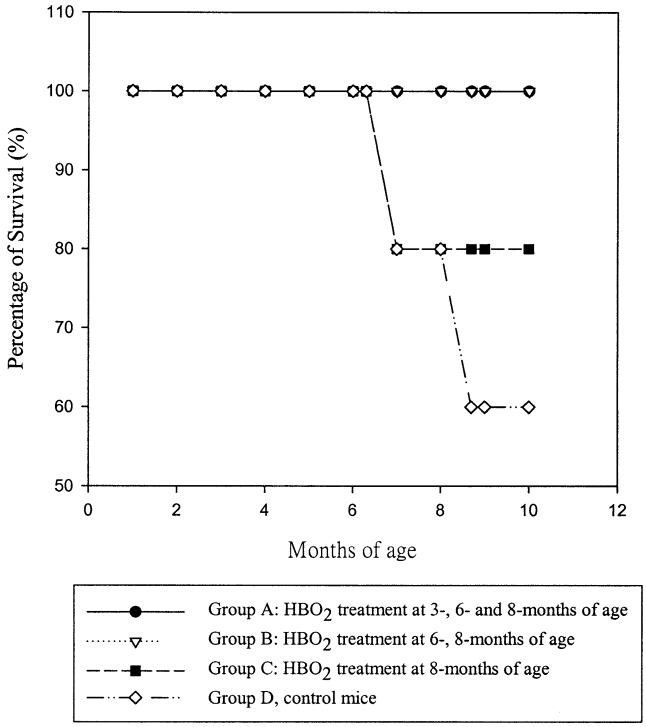

Fig. 1. Comparison of survival curves in group A, B, C, and D mice. Survival rate represents the percentage of mouse survival on individual dates of months.

overnight at 4°C with 10 μg/ml of goat anti-mouse IgG/M (H + L) specific antibody (Jackson ImmunoResearch Laboratories, Inc., USA) diluted in carbonate buffer (pH 9.4). Plates were washed and then blocked with 0.1 ml of 1 mg/ml gelatin–PBS for 2 h at room temperature or overnight at 4°C. After being washed, serum samples (diluted 1:20,000–1:80,000 in gelatin–PBS solution) and a monoclonal mouse IgG or IgM standard (Santa Cruz Biotechnology, USA) were incubated overnight at 4°C. Horseradish-peroxidase-conjugated goat anti-IgM or IgG (Jackson ImmunoResearch Laboratories, Inc.) was added to gelatin–PBS at a 1:5000 dilution, and then incubated for 1 h at room temperature. After three washes with PBS/Tween, the reaction was initiated with 50 ml of 0.55 mg/ml 2.2′-axino-bis-3-ethylbenthiazoline-6-sulfonic acid in citrate buffer (pH 4.2) including 0.03% (v/v) H_2O_2 and terminated by the addition of 0.05 ml of 5% (w/v) SDS. Each sample was tested in duplicate. Background levels were represented by gelatin–PBS and known positive and negative sera were used as controls. Results were read at 420 nm using a SPECTRAmax reader (Molecular Devices Corp.).

Renal histology

Kidneys were removed from mice, snap-frozen in dry ice-cold 2-methylbutane, and embedded in Tissue-Tec (Miles, Elkhart, IN, USA). Freshly cut sections (8 μm) were mounted on clean glass slides coated with poly-L-lysine (Sigma Chemical Co.) and rapidly air-dried and stored at −80°C until used for immunohistochemical staining. The fixed sections were incubated for 15 min at room temperature with normal goat serum diluted 1:5 in PBS to block nonspecific staining. Sections were incubated with FITC-conjugated goat anti-mouse IgG antibody (Chemicon International, Inc., USA) diluted 1:80 in TBS for 1 h at room temperature in a moist chamber and then washed with TBS for 5 min with gentle shaking. After being washed in TBS, they were mounted with Slow Fade reagent (Molecular Probes, Eugene, OR, USA) and covered with a coverslip. Sections of all kidneys were incubated with FITC-conjugated goat anti-mouse IgG antibody. Cross sections of all kidneys were also stained with hematoxylin and eosin. Mesangial hypercellularity was scored blindly by light microscopy as previously described [21].

Statistical analyses

Survival curves were estimated using the Kaplan–Meier method, and the curves were compared using Mantel–Cox analysis. One-way ANOVA and post hoc (Fisher's PLSD) tests were used to compare anti-DNA, serum immunoglobulin concentration, and flow cytometry values.

Results

HBO_2 treatment improved survival rates in group A and B mice

Survival curves are illustrated in Fig. 1. All mice in groups A and B observed till they were 10 months old

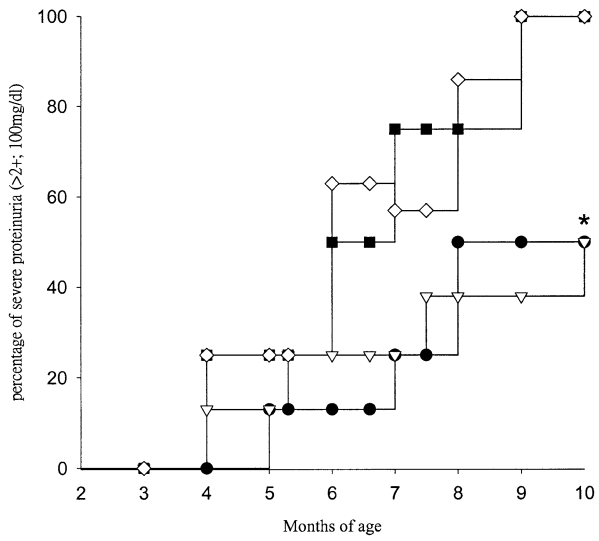

Fig. 2. Cumulative incidence of severe (>2+; 100 mg/dl) proteinuria in group A, B, C, and D of mice. *$P < 0.05$ compared with group D. Urine samples were obtained every 2 weeks for protein measurements by Labstix (Bayer Corp., USA).

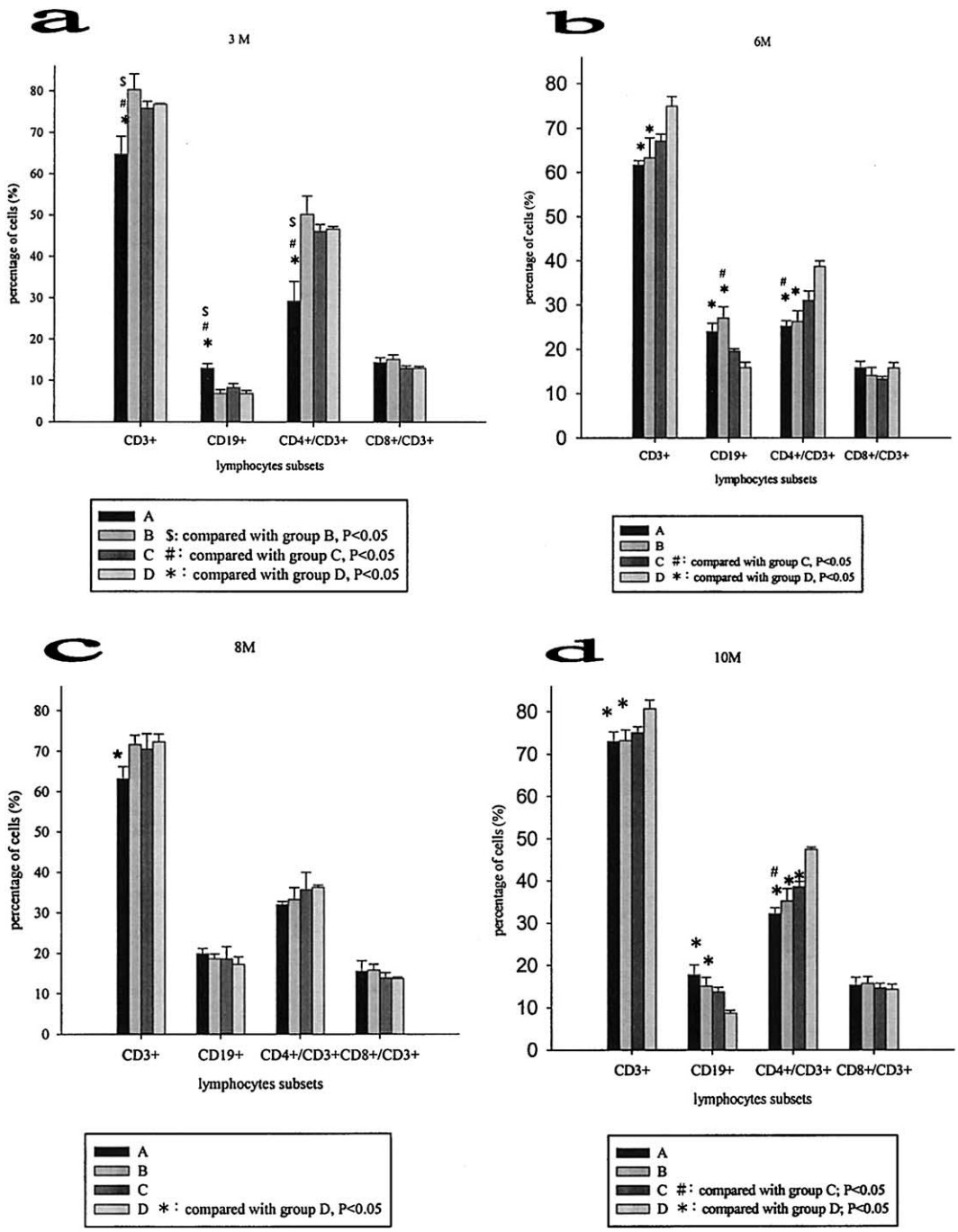

Fig. 3. Flow cytometric analyses of PBMC were performed with CD3, CD19, CD4, and CD8 at (a) 3, (b) 6, (c) 8, and (d) 10 months of age for groups of mice after hyperbaric oxygen treatment. Single cell suspensions of peripheral blood mononuclear cells (PBMC) were prepared in PBS containing 0.1% (w/v) BSA and 0.1% (w/v) NaN_3 (PBS–BSA–NaN_3) and aliquots were individually stained by anti-CD3–FITC, anti-CD19–PE, anti-CD4–PE, and anti-CD8–PE mAbs and analyzed on FACScan.

survived. In contrast, mortality was observed in groups C and D beginning at 9 and 7 months of age, respectively. Our results demonstrated the HBO_2-mediated protection in this autoimmune lupus model. Mice that received earlier treatment showed better protection, suggesting that the therapeutic effect induced by HBO_2 is time-dependent.

The severity of proteinuria was decreased in mice treated with HBO_2

Proteinuria data are illustrated in Fig. 2. Severe proteinuria was defined as >100 mg/dl (>2+). At 4 months of age, 25% of control mice displayed severe proteinuria, and this increased to 50% of control mice at 6 months and

Table 1
Flow cytometric analysis of splenocytes from groups of NZB/WF1 mice at 11 months of age

Group	Splenocytes			
	$CD3^+$ (%)	$CD19^+$ (%)	$CD4^+/CD3^+$ (%)	$CD8^+/CD3^+$ (%)
A	$59.48^{\#*} \pm 3.15$	$24.15^{\#*} \pm 3.55$	$27.86^{\#*} \pm 2.76$	$8.65^{*} \pm 0.59$
B	$64.55^{\#*} \pm 3.95$	$19.94^{\#*} \pm 2.08$	$30.40^{\#*} \pm 3.97$	11.08 ± 1.76
C	75.07 ± 2.05	12.52 ± 1.59	38.75 ± 1.10	13.20 ± 0.57
D	76.80 ± 3.02	9.13 ± 0.96	41.11 ± 2.59	13.55 ± 0.72

$^{\#}$ $P<0.05$ for group A and B mice compared with group C mice.
* $P<0.05$ for group A, B, and C mice compared with control mice.

100% of control mice at 10 months. A similar pattern of proteinuria was seen in group C mice. In contrast, 0, 13, and 50% of group A mice showed severe proteinuria at 4, 6, and 10 months of age, respectively. Mice in group B began to show severe proteinuria (13%) earlier (4 months old) than mice in group A, and 50% of mice in group B had severe proteinuria at 10 months of age. However, this was still reduced when compared to control mice ($P < 0.05$).

Flow cytometric analysis among groups of mice

Flow cytometry analyses of PBMC were performed with CD3, CD19, CD4, and CD8 mAbs at 3, 6, 8, and 10 months of age for all groups of mice. Data were collected after hyperbaric oxygen treatment except from the control mice. FACS analyses of splenocytes were also performed after the mice were sacrificed. According to PBMC analyses of mice at 3 months of age, mice in group A had significantly decreased percentages of $CD3^+$ T lymphocytes and increased percentages of $CD19^+$ B lymphocytes compared to those in the other groups ($P < 0.05$) (Fig. 3a). Among total T lymphocytes, group A mice had markedly decreased subtype $CD4^+$ T cells ($P < 0.05$) compared to those in the other groups. However, there was no significant difference in subtype $CD8^+$ T cells between groups. By 6 and 8 months of age, similar changes in $CD3^+$ T lymphocytes (increased percentage of $CD19^+$ B lymphocytes, decreased percentage of subtype $CD4^+$ T cells) were seen in group A and B mice. Moreover, the percentage of subtype $CD4^+$ T cells in group A mice was significantly less than in group C mice (Fig. 3b and c).

Lymphocyte redistribution of PBMC was also assessed in all mice at 10 months of age. The percentages of $CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, and subtype $CD4^+$ T cells in groups A and B mice were significantly different from those in groups C and D (Fig. 3d). Splenocyte analyses showed similar data, although there was a statistically significant difference in percentage of $CD8^+/CD3^+$ T lymphocytes subsets in group A mice at 11 months of age compared with control mice (Table 1).

Serum levels of anti-dsDNA autoantibody were reduced in HBO_2-treated groups

The serum levels of IgG anti-dsDNA antibody at 3, 6, 8, 10, and 11 months of age among the HBO_2-treated groups and the control group were compared after hyperbaric oxygen treatment (Fig. 4). HBO_2 treatment resulted in decreased serum levels of IgG anti-dsDNA autoantibody in group A mice at 3 months of age compared to control mice ($P < 0.05$), a phenomenon that persisted until the mice were sacrificed. HBO_2 treatment resulted in decreased serum levels of IgG anti-dsDNA antibody in group B mice at 8 months of age and in group C mice at 8 and 11 months of

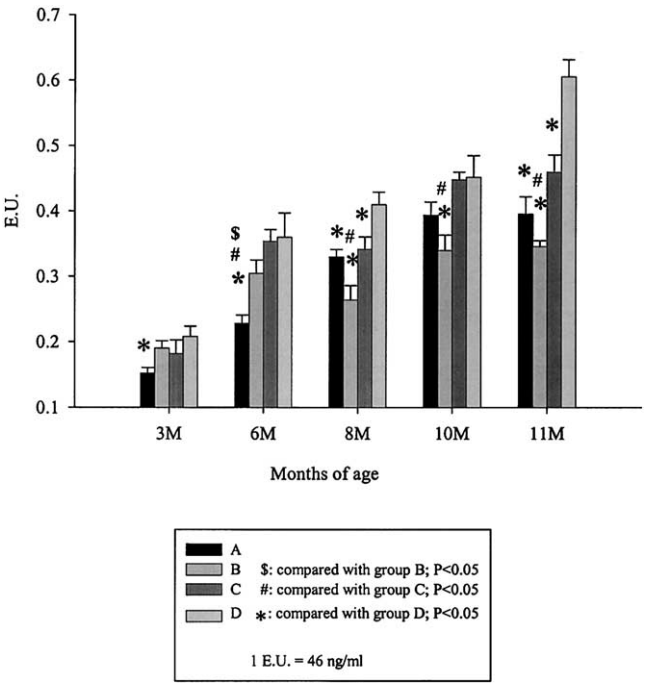

Fig. 4. Comparison of serum levels of IgG anti-dsDNA antibody at 3, 6, 8, 10, and 11 months of age among groups of mice after hyperbaric oxygen treatment. Sera for anti-DNA analysis were collected with nonheparinized tubes using peripheral blood. Standard curve was made by serial dilution of anti-dsDNA autoantibody producing B cell hybridoma supernatant. EU (ELISA unit) was determined by comparing samples with one optimal dilution ratio of hybridoma supernatant.

Table 2
Comparison of total serum IgG/M concentration from groups of NZB/WF1 mice at 3, 6, 8, and 10 months (M) of age

Group	Total IgG (EU)				Total IgM (EU)			
Age:	3 M	6 M	8 M	10 M	3 M	6 M	8 M	10 M
A	2.06 ± 0.41	1.78 ± 0.3	1.66 ± 0.22	1.98 ± 0.66	1.19 ± 0.54	1.39 ± 0.19	1.86 ± 0.86	0.78 ± 0.27
B	1.75 ± 0.54	1.76 ± 0.2	1.97 ± 0.12	1.65 ± 0.11	1.11 ± 0.3	1.29 ± 0.29	2.08 ± 0.79	0.77 ± 0.26
C	1.76 ± 0.17	1.69 ± 0.33	1.63 ± 0.62	1.58 ± 0.46	0.88 ± 0.04	1.09 ± 0.12	1.45 ± 0.41	0.92 ± 0.38
D	1.86 ± 0.25	1.69 ± 0.2	1.93 ± 0.39	1.63 ± 0.38	1.12 ± 0.39	1.2 ± 0.27	1.89 ± 0.75	1.01 ± 0.16

Note. For total IgG: 1 EU (ELISA unit) = 250 ng/ml; total IgM: 1 EU (ELISA unit) = 250 ng/ml.

age. However, there were no difference in serum levels of total IgM/G among all groups of mice (Table 2).

HBO_2 treatment ameliorated the immune-complex deposition and mesangial hypercellularity in group A and B mice

Analysis of renal pathology in the HBO_2-treated groups and the control group was performed at 11 months of age. FITC-conjugated goat anti-mouse IgG antibody was applied to detect glomerular immune-complex deposition. While several glomeruli with strong immunofluorescent staining were detected in group C and D mice, there were very few glomeruli with faint immunofluorescent staining in group A and B mice (Fig. 5A). Mesangial proliferation was also evaluated by hematoxylin and eosin staining of the glomeruli (not shown). Group A and B mice had only mild mesangial hypercellularity compared to group C and D mice (Fig. 5B).

Discussion

The effects of HBO_2 therapy on autoimmune disease have been described previously. However, this is the first study to examine the differential effects of temporal

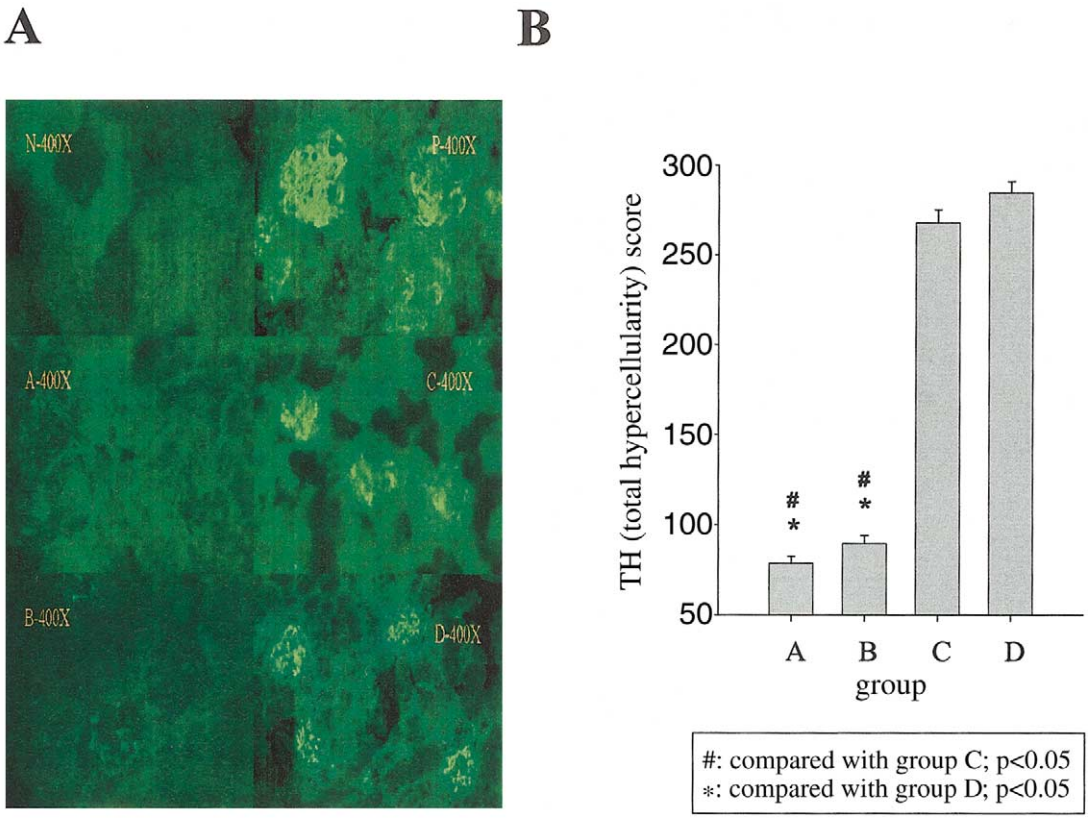

Fig. 5. Renal pathology in the HBO_2-treated groups and the control group at 11 months of age. (A) Sections were incubated with FITC-conjugated goat anti-mouse IgG antibody. Bright staining of glomeruli can be seen on the right (positive control, group C mice, and group D mice from top to bottom) compared to the left (negative control, group A mice, and group B mice). (B) Mesangial hypercellularity of renal tissue was scored blindly by light microscopy as described under Materials and methods.

changes in HBO_2 treatment on lupus-prone mice. In the present study, HBO_2 was initiated in the early, middle, and late stage of clinical disease, as described previously [22], and changes in various disease parameters were assessed. Treatment during the early and middle stages of disease development resulted in significant decreases in proteinuria and increased survival.

Proteinuria in lupus disease results from renal damage secondary to immune-complex deposition. Since T-cells and B-cells play the major roles in the pathogenesis of immune-complex formation in the NZB/W F1 mice [23,24], their distribution as well as anti-dsDNA autoantibody production was examined. HBO_2 treatment suppressed autoantibody production in mice treated with HBO_2 at 3 and 6 months of age.

Long-term (2 months) HBO_2 treatment has been performed in other murine lupus models, such as NZB and MRL/lpr mice. Although total immunoglobulin production was suppressed in both strains, there were no changes in anti-nuclear autoantibody levels [17], which is inconsistent with our observations. However, the animal models used in previous studies have critical differences in disease pathogenesis and phenotype when compared to our model. NZB mice are characterized by an anti-erythrocyte and anti-ssDNA type hemolytic anemia, [18,25], and MRL/lpr mice have a defect of Fas receptor that causes lymphoproliferation and accelerated autoimmunity [26,27]. Thus, the temporal pattern of disease development may differ between these models, yielding different end results following HBO_2 exposure.

Autoreactive $CD4^+$ T cells were recognized as a key factor in the pathogenesis of NZB/W F1 mice [23,28]. $CD4^+$ T cells can induce B cells to secrete cationic IgG anti-dsDNA antibodies and also enhance autoreactive B cells to produce high-affinity pathogenic autoantibodies [29,30]. FACS analysis of PBMC and splenocytes demonstrated a reduced percentage of $CD4^+$ T lymphocytes in NZB/W F1 mice exposed to early and middle HBO_2 treatment. It is possible that autoantibody suppression is secondary to decreased autoreactive $CD4^+$ T cells and/or directly decreased autoreactive B cells, although the percentage of B cells was increased in the current study. Thus, the issue would benefit from further study using an in vitro culture system with individual autoreactive lymphocytes subsets.

Several mechanisms have been proposed for HBO_2-induced immunosuppression, including direct oxygen cytotoxicity or indirect secretion of oxidative-stress-inducing endogenous steroid hormone that leads to generalized lymphocyte death or dysfunction [17]. However, the present study suggests that HBO_2-mediated immunosuppression targets a particular cell population, specifically autoreactive lymphocytes. Oxidative stress and free radicals, such as superoxide ($O_2^{-\cdot}$) and hydroxyl radicals ($OH^{\cdot}$), can be produced by HBO_2 activating transcription factors such as NF-κB, resulting in increased apoptosis [31,32]. We are currently applying the HBO_2 to the splenocytes from NZB/W F1 mice in vitro. Significant apoptotic cells were found in HBO_2-treated groups, compared to those in HBA (hyperbaric air)-treated groups (our unpublished data). Future experiments will be needed to examine whether autoreactive lymphocyte populations in murine lupus are more susceptible to oxidative stress or free radicals.

Renal histopathology in our studies was consistent with proteinuria and anti-nuclear antibody (ANA) production data. Few deposits of immune complexes were demonstrated in group A and B mice. Furthermore, even group A and B mice with severe proteinuria and high ANA titer still did not evince the frequency of glomeruli deposits seen in group C and control mice. Although our experiments did not assess whether the attenuation of lupus nephropathy was the result of early exposure of HBO_2 and/or accumulative effect of HBO_2 treatment, HBO_2 therapy did inhibit the progression of renal complex deposition.

Several classes of drugs, such as steroids, nonsteroid anti-inflammatory drugs, antimalarials, and immunosuppressive reagents are currently used for systemic lupus erythematosus patients. However, significant side effects from medical therapy still pose considerable difficulties. The current study investigated the efficacy and differential effects of HBO_2 therapy applied at different time periods in disease progression in an animal model. We conclude that HBO_2 therapy has potential therapeutic applications in the clinical treatment of lupus disease progression.

Acknowledgments

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