Hyperbaric oxygen inhibits venous neointimal hyperplasia following arteriovenous fistulization

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Abstract. Hypoxia following arteriovenous fistulization results in venous neointimal hyperplasia (VNH), potentially causing early arteriovenous fistula (AVF) dysfunction. In this study, we used hyperbaric oxygen (HBO) in a rabbit model of AVF to determine whether it could ameliorate early AVF failure. Chronic renal failure was induced by adenine in 96 adult rabbits randomly divided into 3 groups (n=32 in each group). The sham + HBO group underwent sham operation and received HBO. The AVF alone group underwent fistulization, but did not receive HBO. The AVF + HBO group underwent fistulization and received HBO. Each group was further divided into 4 subgroups of 8 rabbits each that were euthanized at 1, 7, 14 or 28 days post-operatively. At each time point, blood flow changes in the AVF venous segment were detected using a high-frequency duplex ultrasonography system. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA), and hematoxylin and eosin staining were performed to evaluate VNH. Western blot analysis was performed to confirm the expression of hypoxia-inducible factor (HIF)-1α. At 14 and 28 days following HBO treatment, blood flow in the AVF + HBO group was greater than that at day 0. The AVF + HBO group had a smaller ratio of intima to media area, a lower HIF-1α protein expression, and a smaller percentage of PCNA-positive cells in the proximal vein than did the AVF alone group. Our results thus suggest that continuous HBO treatment following AVF significantly inhibits VNH and promotes blood flow. Therefore, early AVF failure may be prevented by the use of HBO therapy.

Introduction

The arteriovenous fistula (AVF) is the preferred hemodialysis vascular access for patients with end-stage renal disease (ESRD) (1). However, it has been reported that early AVF failure occurs in 28-60% of AVFs, in which the AVF fails to mature sufficiently to support dialysis therapy (2,3). Early AVF failure is defined by venous neointimal hyperplasia (VNH) in combination with a fistula with inadequate blood flow to support dialysis and of insufficient size to allow for repetitive cannulation, caused by inadequate vascular dilatation (4). Although the molecular mechanisms underlying VNH development are multifactorial, previous studies have suggested that hypoxia plays a key role (5,6). Hypoxia is known as the primary stimulus enhancing the expression of hypoxia inducible factor (HIF)-1α, which is a transcription factor regulating numerous genes, including vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) (7). The interaction between VEGF and the VEGF receptor is an essential step in angiogenesis, cellular proliferation, sprouting and migration. MMPs are key enzymes that cause the breakdown of extracellular matrix proteins, such as collagen and elastin, which facilitate the migration of vascular smooth muscle cells (VSMCs) in VNH (8). Misra et al found that the protein levels of HIF-1α, pro-MMP-2 and pro-MMP-9 were significantly increased in specimens removed from patients with failed hemodialysis vascular accesses and in a porcine model of hemodialysis graft failure. They also found that HIF-1α, VEGF-A and MMP-2 gene expression was significantly upregulated at the venous stenosis in a mouse model of AVF with renal insufficiency (5,6). Collectively, these observations suggest that hypoxia and HIF-1α play an important role in neointimal formation in early AVF failure.

Therapy aimed at improving oxygen tension in the vascular wall may prevent early AVF failure by reducing HIF-1α expression. Previous studies have demonstrated that artery wall hypoxia, neointimal formation and VSMC proliferation can be inhibited by the administration of 40% supplemental oxygen in an artery-to-prosthetic graft anastomosis model. Moreover, 42 days of 30% supplemental oxygen was shown to attenuate
All rabbits were provided daily with standard pelleted rabbit chow containing adenine, which they ate *ad libitum*. The concentration of adenine in the chow was 0.6% for the first 4 weeks, followed by 0.3% for 2 weeks (induction phase), and 0.15% (maintenance phase) thereafter, until the termination of the study. Rabbits had free access to tap water throughout the experiment. Blood samples were obtained from the auricular vein before (week 0) and every week after the first adenine feeding until the end of the experiment. Red blood cell (RBC) and Hb values were determined using a hematology analyzer (Sysmex XE-5000; Sysmex, Kobe, Japan). The levels of serum creatinine (SCr) and blood urea nitrogen (BUN) were measured using an automatic biochemical analyzer (Roche, Basel, Switzerland).

**Creation of a rabbit model of CRF.** The primary aim of this study was to examine the hypothesis that HBO therapy may inhibit the expression of HIF-1α and proliferating cell nuclear antigen (PCNA), attenuate tissue and perfused with 0.9% NaCl solution at 80 mmHg for 2 min via an abdominal aortic cannulation and cut off 1 cm from the proximal vein after surgery (on days 0, 1, 7, 14 and 28).

**High-frequency ultrasonography.** A high-frequency duplex ultrasonography system (GE LOGIQ-P5; GE Healthcare Piscataway, NJ, USA) and a 7.5-MHz transducer were used to detect changes in vascular morphology and hemodynamics in the proximal vein after surgery (on days 0, 1, 7, 14 and 28). The proximal vein was imaged within ~1 cm of the fistula in the longitudinal plane. After the vascular longitudinal and transverse axes views were scanned, the average diameter of the vessels was measured using two-dimensional echocardiography, the velocity time integral (VTI) of flow and heart rate (HR) were recorded using the pulsed Doppler technique, and blood flow (ml/min) was calculated using the formula π(AD/2)^2 x VTI x HR.

**Materials and methods**

**Study design.** Study approval was obtained from the Institutional Animal Care and Use Committee of Chongqing Medical University, Chongqing, China before any procedures were performed on the animals. A total of 96 adult New Zealand white rabbits, with an average weight of 3,000 g, were maintained in separate cages at constant humidity and temperature. The animal room was kept on a 12-h light/dark cycle. The rabbits were anesthetized with pentobarbital, and vessels were dissected free of the surrounding soft tissue and perfused with 0.9% NaCl solution at 80 mmHg for 2 min via an abdominal aortic cannulation and cut off 1 cm from the proximal vein after surgery (on days 0, 1, 7, 14 and 28).

**Tissue harvesting.** The rabbits were anesthetized with pentobarbital, and vessels were dissected free of the surrounding soft tissue and perfused with 0.9% NaCl solution at 80 mmHg for 2 min via an abdominal aortic cannulation and cut off 1 cm from the proximal vein after surgery (on days 0, 1, 7, 14 and 28).

**HBO therapy and blood gas analysis.** A total of 5 days of HBO treatment followed by 2 days of rest was defined as the course of treatment. Four such courses were performed, totaling 28 days. The first session began on the first post-operative day with 8 animals at a time. A hyperbaric chamber was pressurized to 2.5 ATA over a period of 20 min. The inlet and vent valves were adjusted to maintain the oxygen pressure (2.5 ATA) and concentration (97-100%) for 60 min and then slowly depressurized over a period of 20 min. Blood pH, pO2, pCO2, and HCO3 values were determined using a blood-gas analyzer (Ciba Corning Diagnostics Corp., East Walpole, CA, USA). Blood samples were drawn via an ear artery catheter during HBO treatment sessions.

**Creation of a carotid-jugular model of AVF.** In the sixth week, arteriovenous fistulization was performed at the left neck. Under x10-loupe magnification, the operative field was sterilized with povidone-iodine and isolated with sterile field clots. A 3-cm skin incision was made along the left mandibular angle to the left medial clavicle. The areas proximal and distal to the left common carotid artery (LCCA) and the left common jugular vein (LCJV) were exposed and clamped with two vascular clamps. An arteriotomy was subsequently performed at the LCCA (~3-mm incision). The LCJV was used for a side-to-side anastomosis to the LCCA using a 9-0 Prolene suture, and the distal side of the LCJV was permanently ligated using a 5-0 silk suture. The AVF was created when the anastomotic vein exhibited stable tremors and pulsation (Fig. 1).

**HBO therapy** with hyperbaric oxygen (HBO) therapy was defined as the intermittent inhalation of 100% oxygen inside a chamber with pressure greater than 1 ATA (12). Ritts et al found that HBO can decrease intimal thickness following carotid artery balloon injury in a rat model (13). Sharifi *et al* concluded that HBO inhibits human coronary artery restenosis following interventional surgery (14). However, these studies primarily focused on arterial disease, while the effect of HBO on venous disease, specifically on early AVF failure, has not yet been investigated, at least to the best of our knowledge.

The primary aim of this study was to examine the hypothesis that HBO therapy may inhibit the expression of HIF-1α and proliferating cell nuclear antigen (PCNA), attenuate venous disease, and further increase blood flow in the venous segment in a rabbit model of AVF with chronic renal failure (CRF).
the fistula in the longitudinal plane. Subsequently, after being separated from the arteries, the venous segments were cut into two segments, one of which was frozen in liquid nitrogen for western blot analysis and the other fixed in 4% paraformaldehyde for immunohistochemical analysis and histological staining.

*Hematoxylin and eosin (H&E) staining.* The fixed venous segments in each group were embedded in paraffin, cut into 5-µm-thick sections, and stained with H&E. VNH was assessed using Image Pro Plus (IPP) 6.0 (Media Cybernetics, Atlanta, GA, USA). VNH was reported as the average intima thickness, intima area, and the ratio of the intima and media areas. Ten sections from each animal were analyzed by 3 experimentally blinded investigators, and the values were averaged.

*Immunohistochemistry.* PCNA highly correlates with cell division (15). Immunohistochemical staining for PCNA was used to assess cell proliferation in neointimal formation. Slides of the veins from each group were deparaffinized with xylene, rehydrated with a gradient of ethanol, blocked in 3% H$_2$O$_2$ at 37˚C for 15 min, and then rinsed 3 times with phosphate-buffered saline (PBS) for 5 min each. Antigen retrieval was performed by bringing the slides to a boil in a sodium citrate buffer, then maintaining a sub-boiling temperature for 20 min. The cooled slides were blocked by serum albumin in an incubator at 37˚C for 30 min, incubated with mouse polyclonal PCNA antibody (1:200 dilution; ab19166; Abcam, Cambridge, UK) at 4˚C overnight, washed with PBS, incubated with biotin-labeled secondary antibody (ab150077; Abcam) at 37˚C for 30 min, and later stained with 3,3'-diaminobenzidine. Samples were counterstained with hematoxylin, dehydrated with xylene and sealed with neutral resins. The percentage of PCNA was used to represent cellular proliferation. All nuclei (denominator) and PCNA-stained nuclei (numerator) were counted using IPP software to determine the percentage of PCNA-positive cells.

*Western blot analysis.* The total protein from the isolated veins was extracted in a radioimmunoprecipitation assay buffer and quantified using a bicinchoninic acid kit. Equivalent amounts of sample were loaded into each well, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose membranes, blocked for 1 h with 5% dry milk/bovine serum albumin, and immuno-blotted with mouse monoclonal anti-HIF-1α primary antibody (1:1,000 dilution; ab1; Abcam) overnight at 4˚C. Afterward, the membranes were washed with 0.1% Tris-buffered saline solution with 0.05% Tween-20 (TBST) 3 times for 10 min each and incubated with HRP-conjugated anti-mouse secondary antibody (ab205719; Abcam) at 37˚C for 1 h and then washed 3 times prior to band detection using enhanced chemiluminescence on a UVP gel imaging system (UVP, Upland, CA, USA). The relative abundance of protein was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

*Statistical analysis.* All values are expressed as the means ± standard deviation. An analysis of variance was used for comparisons among the 3 groups, and a two-tailed Student’s t-test was used for comparisons between 2 groups or in the same subgroup. A significance level of $\alpha=0.05$ was used.

**Results**

*Models of CRF and AVF.* There was a significant increase in the levels of Scr and BUN during the induction phase; however, these levels stabilized and were essentially unaltered during the maintenance phase. Specifically, the levels of Scr and BUN were significantly increased at the fourth week, and they continually increased to ~3-fold greater than the normal range at the sixth week, suggesting that a successful model of CRF was developed (Fig. 2). The average levels of RBCs and Hb were notably decreased at the sixth week and were stabilized by the end of the experiment (Fig. 3). No rabbits died due to adenine administration or HBO treatment.

*HBO improves paO$_2$.* During HBO administration, paO$_2$ and paCO$_2$ in both HBO-treated groups were significantly increased at the fourth week, and they continually increased to ~3-fold greater than the normal range at the sixth week, suggesting that a successful model of CRF was developed (Fig. 2). The average levels of RBCs and Hb were notably decreased at the sixth week and were stabilized by the end of the experiment (Fig. 3). No rabbits died due to adenine administration or HBO treatment.

*HBO increases blood flow in the proximal vein.* The comparison of blood flow was performed within subgroups before and after HBO treatment. At 14 and 28 days after HBO treatment, blood flow in the AVF + HBO group was increased significantly compared with blood flow on day 0 (day 14,
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Table I. Arterial blood gas parameters in rabbits during hyperbaric oxygen treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>PaO$_2$ (mmHg)</th>
<th>PaCO$_2$ (mmHg)</th>
<th>HCO$_3^-$ (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + HBO (n=32)</td>
<td>7.25±0.02</td>
<td>995.29±59.57$^a$</td>
<td>37.94±7.00$^a$</td>
<td>19.19±1.11</td>
</tr>
<tr>
<td>AVF alone (n=32)</td>
<td>7.24±0.05</td>
<td>81.18±12.35</td>
<td>29.04±5.40</td>
<td>19.48±3.20</td>
</tr>
<tr>
<td>AVF + HBO (n=32)</td>
<td>7.24±0.02</td>
<td>1001.46±53.53$^a$</td>
<td>36.25±7.71$^a$</td>
<td>19.20±1.96</td>
</tr>
<tr>
<td>Reference values</td>
<td>7.31±0.09</td>
<td>96.31±1.24</td>
<td>33.05±2.18</td>
<td>20.60±9.14</td>
</tr>
</tbody>
</table>

$^a$P<0.05 compared with the AVF alone group. Reference values were obtained from normal rabbits prior to adenine administration. HBO, hyperbaric oxygen; AVF, arteriovenous fistula; PaO$_2$, partial pressure of oxygen; PaCO$_2$, partial pressure of carbon dioxide.

HBO attenuates neointimal formation. There was no neointimal formation in the veins of the animals in the sham-operated group throughout the experiment. On day 1, a change in intimal thickness was not measurable with IPP software. The average intimal thickness was 0.0495±0.0081 mm in the AVF alone group and 0.0482±0.0077 mm in the AVF + HBO group on day 7. On day 14, the average intimal thickness was 0.1869±0.0456 mm in the AVF alone group and 0.1089±0.0499 mm in the AVF + HBO group (P<0.05). By contrast, on day 28, the average intimal thickness was 0.2091±0.0296 mm in the AVF + HBO group compared to 0.3488±0.0693 mm in the AVF alone group (P<0.05), which indicates a reduction of 40.1% in the AVF + HBO group (Figs. 5 and 6A).

On day 7, a change in intimal area was not measurable with IPP software. On day 14, the intimal area was 1.06 mm$^2$ in the AVF alone group compared to 0.69 mm$^2$ in the AVF + HBO group.
By contrast, on day 28, there was a statistically significant 35.3% decrease in the intimal area in the HBO group when compared to the intimal area in the AVF alone group (0.99±0.077 vs. 1.53±0.22 mm², P<0.05) (Figs. 5 and 6B).

On days 14 and 28 after HBO treatment, the ratio of the intima and media areas in the AVF + HBO group was significantly decreased compared to that in the non-HBO treated group (Figs. 5 and 6C).

Figure 4. Effect of hyperbaric oxygen treatment on blood flow on days 1, 7, 14 and 28. (A and B) Changes in blood flow on days 1 and 7. (C and D) Changes in blood flow on days 14 and 28. *Blood flow in the arteriovenous fistula + hyperbaric oxygen (AVF + HBO) group was significantly increased on days 14 and 28 after HBO treatment when compared with that on day 0. †Blood flow in the AVF alone group was significantly decreased on days 14 and 28 when compared with that on day 0. Both P<0.05.

Figure 5. Effect of hyperbaric oxygen treatment (HBO) on venous neointimal hyperplasia (VNH) of proximal vein of arteriovenous fistula (AVF). (A and B) Hematoxylin and eosin (H&E) staining of vessel in AVF alone group on post-operative day 28. (C and D) Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in AVF alone group on day 28. (E and F) H&E staining of vessel in AVF + HBO group on day 28 after HBO administration. (G and H) Immunohistochemical staining for PCNA in AVF + HBO group on day 28 after HBO administration. Panels A and B, and panels E and F, original magnification, x100 and x200, respectively. Panels C and D, and panels G and H, original magnification, x200 and x400, respectively.
HBO downregulates PCNA expression. In the sham-operated group, there was a very weak nuclear expression of PCNA throughout the experiment, with no statistically significant differences. On day 1, there was no statistically significant difference in the percentage of PCNA-positive cells among the 3 groups. However, on day 7, compared with the sham-operated group, the number of PCNA-positive cells increased in the 2 AVF groups, with no significant difference. On days 14 and 28, compared with the non-HBO treated group, the percentage of PCNA-positive cells was decreased in the AVF + HBO group (Figs. 5 and 7).

HBO downregulates the protein expression of HIF-1α. In the next experiment, we further examined HIF-1α protein expression by western blot analysis. Arteriovenous fistulization significantly upregulated HIF-1α protein expression in the 2 AVF groups. However, on days 7, 14 and 28 after HBO
Discussion

AVF has been referred to as the 'lifeline' of patients with ESRD. However, due to early AVF failure resulting from VNH, reduced blood flow, and even thrombosis and occlusion, dialysis therapy cannot be carried out on a regular treatment schedule for many patients. The effective treatment and prevention of VNH in clinical practice have continued to elude physicians. Strategies to prevent VNH, including pharmacological therapy (16), brachytherapy (17) and even gene-directed therapy (18), have yet to be widely used clinically. This study demonstrated that HBO inhibited VNH and increased blood flow in the AVF in a rabbit model of CRF.

Previously, the majority of studies on hemodialysis vascular access were performed on animal models with normal kidney function, which does not adequately simulate the clinical scenario. It is well recognized that ESRD is associated with increased oxidative stress and chronic inflammation. Nitrotyrosine and peroxynitrate, products of oxidative stress, increase the expression of VEGF and MMPs (19). Inflammatory blood markers, such as high-sensitivity C-reactive protein, interleukin-6 and tumor necrosis factor-α, are associated with the magnitude of VNH and the development of thrombosis in early AVF failure (20). A previous study by Yokubo et al concluded that chronic kidney disease accelerated the development of neointimal hyperplasia at the anastomotic site of an AVF (21). In this study, the SCr and BUN levels in all rabbits increased at the fourth week after adenine administration. At the sixth week, the SCr and BUN levels continued to increase, to ~3-fold greater than the normal range, and they then plateaued and remained stable until 10 weeks. Moreover, decreases in the RBC and Hb counts were observed. These pathological changes occurred in rabbits that successfully modeled the clinical course of progressive human kidney disease. We consider that this model is particularly suitable for testing HBO treatment methods, due to the fact that the reduction in paO₂ in animals with CRF may be ameliorated by HBO without the need for Hb. Additional advantages of our model include small inter-individual variation in renal function and zero mortality, limiting the number of animals required for induction.

There are two factors required for AVF maturation. First, the AVF should have adequate blood flow to support dialysis; second, it should have sufficient size to allow successful repeated cannulation. Accordingly, increasing the blood flow and vascular diameter of the AVF as treatment targets will improve the maturation rate of AVF. VNH is the common factor influencing both blood flow and vascular diameter, and it plays an important role in early AVF failure, which results from the abnormal migration and proliferation of VSMCs together with matrix deposition in the venous segments. HIF-1α is expressed in all nucleated cells and functions as a master regulator of gene transcription, mediating cellular homeostatic responses to altered oxygenation (22), whose expression increases exponentially as oxygen concentration declines. HIF-1α has been reported to be a potent regulator of VEGF, MMPs and other genes that have been implicated in VNH (5,7). In our study, HIF-1α protein expression was observed in the AVF alone and AVF + HBO group on days 7, 14 and 28 after AVF placement, with no obvious expression observed in the sham-operated group. These data suggest that surgical trauma will result in hypoxia at the venous wall, consistent with previous findings showing increased hypoxic levels in the vascular segments in animal models of prosthetic vascular graft to artery anastomosis (23). The outer two-thirds of the vascular wall is supplied with oxygen via the vasa vasorum, although the inner one-third of the vascular wall is supplied via the luminal diffusion of oxygen (24). One possible reason for vascular hypoxia is the inevitable damage to vascular endothelial cells and the vasa vasorum due to the incision and suture of the vascular wall and adventitia dissected in the surgical process, which prevents the diffusion of blood from the luminal side and adventitial surface. Additionally, under conditions of uremia, occlusive diseases of the vasa vasorum and endothelial cell dysfunction further exacerbate hypoxia in the vascular wall (25). The present study also demonstrated that the number of PCNA-positive cells in venous segments increased between days 7 and 28, when the venous wall is most hypoxic, and the greatest change in intimal area occurred between days 14 and 28. Previous studies have reported that the release of VEGF, MMPs, platelet-derived growth factor, and fibroblast growth factor increased when endothelial cells or VSMCs were under hypoxic conditions (9,26,27). Further research revealed that the expression of these factors may be upregulated by the HIF-1α-dependent signaling pathway, causing cellular proliferation and migration (28,29). A cascade of events then occurs, whereby vascular wall hypoxia can continue to incite cellular activity, such that the vessel becomes occluded secondary to intimal hyperplasia.

The studies by Wan et al (9), Lata et al (10) and Santilli et al (30) demonstrated that improvement in inhaled oxygen concentration under normal atmospheric pressure can reverse hypoxia and intimal hyperplasia of the vascular wall in animal models of AVF with normal renal function (9,10,30). However, the CRF model has lower arterial oxygen capacity due to anemia, which differs from the models with normal renal function. Therefore, HBO may have an advantage in reversing tissue hypoxia due to its ability to increase the arterial oxygen content and paO₂ by increasing the dissolved oxygen concentration in the plasma. The increased paO₂ improves the driving force and the distance of diffusion in tissues. In this study, the RBC count and Hb level were decreased in adenine-induced CRF, resembling the clinical characteristics of uremic patients. HBO treatment notably increased the paO₂ ~10-fold higher than that in the non-HBO treated group. The AVF-induced expression of HIF-1α and PCNA was significantly attenuated by treatment with HBO for 28 days. These results suggest that the administration of HBO may improve oxygen levels, resulting in the inhibition of HIF-1α expression and reduced cellular proliferation in the proximal venous segments. Moreover, HBO therapy did result in a statistically significant decrease in average intimal thickening by 40.1% and intimal area by 35.3% following AVF placement. Blood flow in the venous segments increased along with the reduction of VNH. These results confirm our hypothesis that continuous exposure to HBO can significantly inhibit VNH and improve blood flow following AVF placement.

There are a few limitations to the present study that should be mentioned. Our model primarily reflects a tubulointerstitial
References


