Supplemental oxygen reduces intimal hyperplasia after intraarterial stenting in the rabbit

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Hypothesis: Supplemental oxygen can reduce intimal hyperplasia (IH) after stent deployment in a rabbit model.

Background: Endovascular stent placement is technically feasible, but long-term durability in vessels outside the aortoiliac system is compromised with postinterventional IH, which causes restenosis and failure of the arterial conduit.

Methods: Groups (n = 4 to 6) of female New Zealand white rabbits underwent placement of a 3-mm intraaortic stent with laparotomy and were placed in either normoxic (21% inspired oxygen concentration) or supplemental-oxygen (40% inspired oxygen concentration) environments for 0, 7, 14, and 28 days. The transarterial wall oxygen gradient was measured at 0, 7, and 28 days with an oxygen microelectrode. 5-Bromo-2’deoxyuridine (BrdU) was injected into the peritoneum before death to assess cellular proliferation. Aortic specimens were harvested en bloc and sectioned for analysis of cellular proliferation and intimal thickness.

Results: Intraaortic stent placement significantly decreased the transarterial wall oxygen gradient in the outer 70% of the vessel wall and was easily reversed at 7, 14, and 28 days with application of supplemental oxygen. Cellular proliferation was significantly decreased at 14 days (0.5% ± 0.001% versus 2.3% ± 0.002%; P < .001) and 28 days (0.4% ± 0.001% versus 1.0% ± 0.001%; P < .025) as measured with count of nuclei staining for 5-Bromo-2’deoxyuridine in the intima and media. Intimal thickness was significantly decreased at 28 days in oxygen-supplemented rabbits (intimal area/medial area = 0.50 ± 0.07) as compared with controls (intimal area/medial area = 0.89 ± 0.11; P < .025).

Conclusion: This study shows the ability of supplemental oxygen to reverse arterial wall hypoxia, decrease cellular proliferation, and control IH at the deployment site of an intraarterial stent in a rabbit model. Forty-percent supplemental oxygen suppresses IH by 44% at 28 days as compared with normoxic control values. Cellular proliferation is reduced four-fold at 14 days and two-fold at 28 days in oxygen-supplemented rabbits as compared with control media after deployment. The clinical implications of these findings are significant, especially as the role of endovascular interventions continues to expand. (J Vasc Surg 2002;35:982-7.)

Postinterventional injury to arterial endothelium results in intimal hyperplasia (IH) and is common after arterial interventions, including bypass grafting, endarterectomy, angioplasty, and stent placement for atherosclerotic occlusive or atherosclerotic disease. Narrowing of the arterial conduit ensues, which leads to ischemia. In the clinical setting, the result may be infarction, stroke, limb loss, and other end-organ damage.

Arterial stent placement and minimally invasive endovascular techniques continue to gain popularity.1-4 Stent placement is generally well-tolerated in large vessels, such as the abdominal aorta and iliac arteries, but long-term durability is often compromised by IH in smaller vessels, such as coronary, carotid, and superficial femoral arteries.

Angiographic restenosis had been shown to be 25% to 31.6% at 6 months after coronary stent placement5,6 and 35.6% at 1 year after superficial femoral artery stent placement.7

The histologic features of IH are well-defined and begin with smooth muscle migration from the media to the intima in response to arterial injury. Cellular proliferation ensues, with elaboration of extracellular matrix and restenosis.8,9 No widely accepted therapy exists at this time. Much current research is directed at elucidation of the mechanisms of intracellular and intercellular signaling and regeneration pathways involved in the formation of IH.5,6

We have previously described the ability of a 40% supplemental oxygen environment to decrease IH at a prosthetic graft to native artery anastomosis in the rabbit.7 Further, we have shown that placement of an intraarterial stent alters the transarterial wall oxygen gradient, inducing arterial wall hypoxia.8 A method of controlling IH after stent deployment would allow for the expansion of endovascular therapy to small diameter arteries.

The purpose of this study was to examine the effect of supplemental oxygen after deployment of an intraarterial stent. We hypothesized that application of supplemental oxygen can reverse the transarterial wall oxygen gradient after intraarterial stent deployment and that supplemental oxygen can reduce IH after intraarterial stent deployment in a rabbit model.
METHODS

Animal model. Female New Zealand white rabbits (2 to 3 kg; age, 9 to 12 months) from a single vendor were allowed to acclimate for at least 3 days before interventions. Rabbits were caged individually and fed standard chow and water ad libitum. Animals were housed and cared for according to an animal use protocol approved by the Minneapolis Veterans Affairs Medical Center Institutional Animal Care and Use Committee and the University of Minnesota.

After placement of an intraarterial stent, rabbits were randomly assigned to control (normoxic, 21% inspired oxygen concentration) or treatment (supplemental oxygen, 40% inspired oxygen concentration) environments. The treatment environment was maintained with the animals in an oxygen chamber (Plas Laboratories, Lansing, Mich), with constant monitoring of ambient atmospheric oxygen concentration (40%) and humidity (50%). Oxygen-supplemented rabbits were removed to a normoxic environment for brief periods daily (< 1 hour) for chamber maintenance, including bedding, chow, and water changes. Thirty-six animals in seven groups underwent measurement of transarterial wall oxygen gradients, and another 35 animals in seven groups underwent image analysis for assessment of IH and cellular proliferation.

Placement of intraarterial stent. Rabbits underwent anesthesia with ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg) intramuscularly and were blindly intubated with a 3-mm endotracheal tube. Anesthesia was maintained with passive isoﬂurane inhalation (Abbott Laboratories, Chicago, Ill). Penicillin (150,000 U) was administered intramuscularly. A 24-gauge intravenous catheter was placed in a marginal ear vein. With sterile technique, the infrarenal aorta was exposed via midline laparotomy incision; proximal and distal control were established below the renal arteries and above the iliac bifurcation. Care was taken to preserve the vasa vasorum. Heparin sulfate (800 U) was administered intravenously. A transverse aortotomy was created 5 mm above the iliac bifurcation. Next, a 3-mm Palmaz-Schatz stainless steel intraarterial stent (Cordis, Warren, NJ) was loaded onto a 3-mm outer diameter with 2-cm long balloon dilation catheter (Boston Scientiﬁc/Meditech, Watertown, Mass) and inserted proximally via the aortotomy and deployed below the renal arteries with balloon inﬂation to 3 atm for 30 seconds. The catheter was removed, and the aortotomy was closed with 7-0 monofilament sutures.

Perioperative blood loss was replaced with intravenous normal saline solution. Postoperative analgesia (buprenorphine, 0.05 mg/kg) was administered for 48 hours.

Transarterial wall oxygen gradient measurements. After stent placement, animals were placed in the following arms: 1, 0-day control (n = 6); 2, 7-day control (n = 5); 3, 28-day control (n = 5); 4, 0-day treatment (n = 5); 5, 7-day treatment (n = 5); and 6, 28-day treatment (n = 6); for determination of transarterial wall oxygen gradients. An additional control group (n = 4) of 0-day, nonstented, normoxic animals was analyzed. Briefly, with general anesthesia and via midline laparotomy, a previously calibrated oxygen microelectrode was introduced into the midpoint of the 3-mm infrarenal intraaortic stent at the adventitia and advanced toward the lumen. Oxygen-supplemented animals received 40% FiO2 during anesthesia, and control animals received 21% FiO2. Measurements of artery wall tissue oxygen level were recorded at 10-µm increments with a chemical microsensor (Model 1201; Diamond General Corp, Ann Arbor, Mich). Additional details regarding construction and calibration of the electrode are described elsewhere.9,10

Cellular proliferation. 5-Bromo-2’-deoxyuridine (BrdU, Zymed Laboratories, San Francisco, Calif) was used to assess cellular proliferation. BrdU is incorporated into the nuclei of dividing cells. Twenty-four hours before death, BrdU (100 mg/kg; Sigma Chemical Company, St Louis, Mo) was administered via intraperitoneal injection.

Intraaortic stent harvest. Rabbits were killed with a lethal injection of pentobarbital. The infrarenal aorta and the infrarenal vena cava were cannulated with 20-gauge catheters, and the animal was exsanguinated with normal saline solution. Karnovsky’s solution was administered to perfuse the aorta and intraaortic stent at 300 mm Hg pressure for 10 minutes. Samples were harvested en bloc and placed in formalin.

Specimen processing. Formalin-fixed specimens were dehydrated in ascending grades of ethanol (70% to 100%) at 37°C. After dehydration, the specimens were thoroughly infiltrated in methyl methacrylate with a three-step protocol at 4°C in a vacuum chamber. After infiltration with methyl methacrylate, the polymerization process was completed with placement of the specimen in a 37°C oven until hardened. Cutting and grinding through the stented portion of the aorta produced 30-µm to 50-µm ground sections, which were stained with hematoxylin and eosin, air dried, and coverslipped with acrylic mounting medium.

Four-µm aortic cross sections were acquired 1 mm distal to the stent for BrdU staining with the Zymed BrdU staining kit. Sections were mounted on glass slides and dried in a 60°C oven. The sections then were deparafﬁnized in xylene and rehydrated with a series of ethanol. Endogenous peroxidase activity was attenuated in peroxidase quenching solution, and the slides were rinsed in phosphate-buffered saline solution (PBS). Sections then were treated with trypsin for 10 minutes in a moist chamber at 37°C, followed by incubation with denaturing solution to increase accessibility of antigen. Specimens were treated for nonspeciﬁc labeling with blocking solution for 10 minutes. Biotinylated mouse anti-BrdU reagent was applied for 60 minutes, followed by a rinse in PBS. Sections were incubated for 10 minutes with streptavidin-peroxidase. After rinsing, the color was developed with incubation with 3,3’-diaminobenzidine mixture with the aid of a microscope. Sections were counterstained with hematoxylin, washed in a tap water, transferred into PBS until the nuclei were blue, and then rinsed in distilled water. Slides were dehydrated in graded alcohol solutions, cleared in xylene, and coverslipped.
Image analysis. Image analysis was carried out in a blinded fashion. Specimens were analyzed for IH with a microscope with a digitized camera attachment (Zeiss, Oberkochen, Germany) feeding real time images to image analysis software (Bioquant, R&M Biometrics, Nashville, Tenn) on a personal computer. The digital camera was calibrated with measurement of a known distance on the microscope stage. Intimal and medial boundaries then were marked, and intimal and medial areas were automatically calculated. One proximal and one distal section were obtained from each specimen. IH is reported as the ratio of intimal area (IA) to medial area (MA), to standardize for varying animal aortic sizes and extent of fixation.

Cellular proliferation was assessed at 1 mm distal to the stent because the sectioning process for the stented aorta was not compatible with the BrdU assay. Cellular proliferation was evaluated with image analysis software (Bioquant) to count cells with brown stained BrdU nuclei in the intima and media of each specimen, at 1 mm distal to the stent. This process was repeated to obtain a total count of all cells in the intima and media. Cellular proliferation is reported as the ratio of BrdU-stained cells to the total cell count.

Statistical analysis. Transarterial wall oxygen gradients are expressed as mean ± standard deviation. IH and cellular proliferation data are expressed as mean ± standard error of the mean. Student two-tailed t test was used to compare transarterial wall oxygen gradients, IH, and cellular proliferation between rabbits with oxygen supplementation (treatment) and normoxic animals (controls) at varying time points. Normal distribution of the data was determined and confirmed with kurtosis and skewness tests. A P value less than .05 was considered significant.

RESULTS

Placement of an intraarterial stent resulted in immediate arterial wall hypoxia in the outer 70% of the vessel wall as compared with nonstented controls (P < .01; Fig 1) in a normoxic (21% oxygen) setting. This effect was reversed with application of 40% supplemental oxygen, where mean tissue oxygen tensions were increased through the entire artery wall (P < .001; Fig 2). Similarly, supplemental oxygen increased the transarterial wall oxygen gradient in 7-day and 28-day oxygen-supplemented animals when compared with normoxic controls (P < .001; Fig 3). Nadir values occurred at similar sites in each group, with 60% of the vessel wall. In addition, day-0 oxygen-supplemented rabbits had significantly lower transarterial wall oxygen tensions in the outer 80% of the vessel wall (P < .025; Fig 3).

Cellular proliferation was decreased in oxygen-supplemented rabbits at 14 days as compared with normoxic controls (0.5% ± 0.001% versus 2.3% ± 0.002%; P < .001; Figs 4 to 6). A similar effect was appreciated at 28 days, where cellular proliferation was again decreased in the oxygen-supplemented animals (0.4% ± 0.001% versus 1.0% ± 0.001%; P < .025; Fig 7). Cellular proliferation was not significantly different at 7 days.

IH was decreased in the oxygen-supplemented rabbits (IA/MA = 0.50 ± 0.07; Table) as compared with normoxic controls at 28 days (IA/MA = 0.89 ± 0.11; P < .025). No difference in IA/MA was seen in 7-day or 14-day arms. Histologic analysis results showed persistence of vasa vasorum in the periadventitial tissue after stent deployment.

DISCUSSION

This study is the first published report to show the ability of supplemental oxygen to reverse arterial wall hypoxia, decrease cellular proliferation, and control IH at the deployment site of an intraarterial stent in a rabbit model. Forty-percent supplemental oxygen suppresses IH by 44% at 28 days as compared with normoxic control values. Cellular proliferation is reduced four-fold at 14 days and two-fold at 28 days in oxygen-supplemented rabbits as compared with controls. The clinical implications of these findings are significant, especially as the role of endovascular interventions continues to expand.
Delineation of the mechanism of IH formation suggests multiple pathways, mediated by platelets, macrophages, and leukocytes, elaborating growth factors and cytokines.\textsuperscript{5,6,11-13} However, we believe hypoxia should be construed as an inciting event in the development of IH, a signal for the biochemical cascade that characterizes the pathobiology of IH. In these experiments, we were able to show that transarterial wall oxygen profiles were significantly diminished in the outer 70% of the vessel wall immediately after placement of a stent and that this effect could be easily and convincingly reversed with the application of 40% supplemental oxygen at normal atmospheric pressure.

Fig 2. Transarterial wall oxygen gradient for intraarterial stent day-0 rabbits, control versus oxygen-supplemented (mean ± standard deviation), with all values significantly different ($P < .001$). $PO_2$, Partial pressure of oxygen.

Fig 3. Transarterial wall oxygen gradients for intraarterial stent day-7 and day-28 rabbits for control versus oxygen-supplemented (mean ± standard deviation), with all supplemented values significantly higher than control values ($P < .001$). $PO_2$, Partial pressure of oxygen.

Fig 4. Cellular proliferation as measured with ratio of cells with positive staining results for BrdU to total cell count of intima and media for 14-day control and oxygen-supplemented rabbits ($P < .001$).

Fig 5. Cellular proliferation 1-mm distal to stent in 14-day control rabbit. Immunoperoxidase staining of BrdU-labeled cells. Darkly stained nuclei (arrows) represent cells with positive staining results. \textit{IEL}, Internal elastic lamina (biotinylated mouse anti-BrdU; original magnification, ×200).
The exact mechanism for the effect of a supranormal transarterial wall oxygen gradient has not yet been delineated. We hypothesize that the observed inhibition of IH with supplemental oxygen is on the basis of reversal of hypoxia and a reduction in oxidative stress in the vessel wall. Stent deployment is known to decrease the transarterial wall oxygen gradient.\textsuperscript{8} The increased circumferential and radial stress associated with intraarterial stent deployment decreases oxygen delivery and increases oxygen consumption, inducing a hypoxic state.\textsuperscript{8} Percutaneous transluminal coronary angioplasty has been shown to result in depletion of glutathione levels\textsuperscript{14} and production of isoprostanes\textsuperscript{15}; both are indicative of increased oxidative stress. Oxidative stress generates reactive oxygen species, such as the superoxide, hydroxyl, and hydrogen peroxide. Besides their known effects of increased microvascular permeability and tissue damage in models of ischemia/reperfusion and hypoxia,\textsuperscript{16,17} reactive oxygen species can inactivate nitric oxide, a molecule that regulates smooth muscle cell migration and proliferation.\textsuperscript{6,18,19}

Potential shortcomings of this study should be addressed. The open model of stent placement does not duplicate minimally invasive techniques used in humans, but careful intraoperative dissection was performed to preserve the periadventitial tissues and prevent disruption of blood flow to the arterial wall. Evidence of the success of this technique was borne out in histologic examination that confirmed presence of vasa vasorum. This study was performed on healthy rabbit aortas, which is in sharp contrast to the most common clinical scenario of diseased atherosclerotic artery walls. Future studies should address the efficacy of supplemental oxygen in an atherosclerotic model. Oxygen toxicity may be seen at high levels of inspired oxygen. The side effects of supplemental oxygen at low levels seem minimal in man, provided concentrations are kept below 50%.\textsuperscript{18,20} Rabbits in this study did not appear to have adverse consequences from 1 month of a 40% supplemental oxygen environment. High levels of supplemental oxygen (>80%) have been shown to cause adverse clinical consequences in humans, such as pulmonary edema and retinopathy of prematurity and in vitro cellular toxicity.\textsuperscript{16,18,20,21} Natural defense systems, such as superoxide dismutase, catalase, and glutathione peroxidase, may become overwhelmed, and indeed evidence for decreases in enzyme activity and increased reactive oxygen levels have been shown at high levels of oxygen supplementation.\textsuperscript{20,22,23}

Finally, we have shown a reduction of IH at a short-term time point. Long-term outcomes after application of supplemental oxygen will need to be evaluated in a rabbit model. Ultimately, the effectiveness of supplemental oxygen will need to be assessed in human studies.

**CONCLUSION**

Stent durability in small vessels will continue to be compromised until a dependable treatment for restenosis as a result of IH is available. Administration of 40% supplemental oxygen creates a supraphysiologic hypoxic state and seems to counteract the hypoxic effect of stent deployment on the arterial wall, thereby attenuating IH formation. Supplemental oxygen represents a ubiquitous, safe, inexpensive, and easily administered therapy. We have shown a response to supplemental oxygen in the form of decreased indices of IH early in the postinterventional period.

**Table I.** IH: IA/MA

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<th>Control</th>
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<tr>
<td>0 day</td>
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<tr>
<td>7 day</td>
<td>0.61 (± 0.21)</td>
<td>0.65 (± 15)</td>
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<td>14 day</td>
<td>0.57 (± 0.09)</td>
<td>0.68 (± 0.17)</td>
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<tr>
<td>28 day</td>
<td>0.89 (± 0.11)</td>
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*P < .05, control day 0 versus all other groups.
†P < .025, control versus oxygen-supplemented 28 days.
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REFERENCES


