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STEM CELL MOBILIZATION BY HYPERBARIC OXYGEN

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ABSTRACT

We hypothesized that exposure to hyperbaric oxygen (HBO_2) would mobilize stem/progenitor cells from the bone marrow by a nitric oxide ($^{\cdot}NO$) dependent mechanism. The population of CD34+ cells in the peripheral circulation of humans doubled in response to a single exposure to 2.0 atmospheres absolute (ATA) O_2 for 2 hours. Over a course of twenty treatments, circulating CD34+ cells increased eight-fold, although the over-all circulating white cell count was not significantly increased. The number of colony-forming cells (CFCs) increased from 16 $\pm$ 2 to 26 $\pm$ 3 CFCs/100,000 monocytes plated. Elevations in CFCs were entirely due to the CD34+ sub-population, but increased cell growth only occurred in samples obtained immediately post-treatment. A high proportion of progeny cells express receptors for vascular endothelial growth factor-2 and for stromal derived growth factor. In mice, HBO_2 increased circulating stem cell factor by 50%, increased the number of circulating cells expressing stem cell antigen-1 and CD34 by 3.4-fold, and doubled the number of CFCs. Bone marrow $^{\cdot}NO$ concentration increased by 1008 $\pm$ 255 nM in association with HBO_2. Stem cell mobilization did not occur in knock out mice lacking genes for endothelial $^{\cdot}NO$ synthase. Moreover, pre-treatment of wild type mice with a nitric oxide ($^{\cdot}NO$) synthase inhibitor prevented the HBO_2-induced elevation in stem cell factor and circulating stem cells. We conclude that HBO_2 mobilizes stem/progenitor cells by stimulating $^{\cdot}NO$ synthesis.

Key Words: nitric oxide, CD-34, CD-133, CXCR4, c-Kit, colony forming cells, progenitor cells

INTRODUCTION

The goal of this investigation was to determine whether exposure to hyperbaric oxygen (HBO_2) would mobilize bone marrow-derived stem/progenitor cells (SPCs) in humans and animals. Pluripotent SPCs from adults exhibit properties similar to embryonic SPCs, and hold promise for treatment of degenerative and inherited disorders (9, 20). Postnatal neovascularization occurs by sprouting of endothelium from preexisting blood vessels (angiogenesis) and by endothelial SPCs released from the bone marrow that home to foci of ischemia in a process termed vasculogenesis (21). SPCs mobilization from the bone marrow can be stimulated by peripheral ischemia, vigorous exercise, chemotherapeutic agents and hematopoietic growth factors (2, 16, 22, 23, 27, 30, 36). SPCs also can be obtained by direct bone marrow harvesting and *ex vivo* manipulations (10, 28, 32). Hematopoietic SPCs are typically obtained for the purpose of bone marrow transplantation by administration of chemotherapeutic agents and growth factors (36). Utilizing these agents to obtain autologous SPCs for treating disorders such as organ and limb ischemia, and refractory wounds, has been considered but application is thwarted due to risks such as acute arterial thrombosis, angina, sepsis and death (7, 20, 21, 27, 29, 30, 36).

Nitric oxide ($^{\cdot}NO$) plays a key role in triggering SPCs mobilization from the bone marrow via release of the stem cell active cytokine, cKit ligand (stem cell factor, SCF) (1, 8). Because HBO_2 can activate $^{\cdot}NO$ synthase in different tissues, we hypothesized that exposure to HBO_2 may stimulate SPCs mobilization to the peripheral circulation (33, 34). In a murine model, we found HBO_2 augments SPCs mobilization, recruitment to ischemic wounds, and hastens ischemic wound healing (submitted[1]).

1- Goldstein LJ, Gallagher K, Baireddy V, Bauer SM, Bauer RJ, Buerk DG, Thom SR, Velazquez OC. Progenitor cell release into circulation is triggered by hyperoxia-induced increases in bone marrow nitric oxide. Submitted.

SPCs have been shown to home to ischemic wounds, where they are required for angiogenesis (3).

HBO_2 therapy is administered for a variety of maladies in a hyperbaric chamber where patients breathe pure O_2 at partial pressures up to 3.0 atmospheres absolute (ATA). HBO_2 is used in a standard fashion as prophylactic treatment to reduce the incidence of osteoradionecrosis (ORN) in patients who must undergo surgery involving tissues previously exposed to radiotherapy (6, 15). We obtained peripheral blood samples from normal human volunteers and from a group of patients undergoing prophylactic HBO_2 in anticipation of surgery to reduce their risk for ORN, and examined the blood for the presence of SPCs. We then investigated the mechanism for SPCs mobilization in mice. Here, we demonstrate that HBO_2 causes rapid SPCs mobilization in both humans and mice, and that this occurs via a $^{\cdot}NO$ dependent mechanism.

METHODS

Stem cell release in humans exposed to HBO_2: This protocol was approved by the Institutional Review Board and by the Clinical Trials Scientific Monitoring Committee of the Abramson Cancer Center. Patients are referred to the University of Pennsylvania Institute for Environmental Medicine for prophylactic HBO_2 treatment due to a risk for ORN. A group of these patients was approached and after informed consent, blood was obtained before and after their first, 10th and 20th HBO_2 treatment (2.0 ATA O_2 for 2 hours). All of these patients had undergone radiotherapy for head or neck tumors, none had open ulcerations, nor were they taking corticosteroids or chemotherapeutic agents. Based on current standard of care they

received HBO_2 therapy prior to undergoing oral surgery due to radiation-induced xerostomia and caries. Men (n=18) had an average age of 56 $\pm$ 2 (SE) years and women (n=8) were 53 $\pm$ 4 years old. Three inside-chamber paramedic attendants, men with an average age of 48 $\pm$ 3 years, also had blood drawn before and after pressurization to 2.0 ATA for 2 hours. These individuals served as a control for the effect of pressure versus hyperoxia, as they breathe air and not pure oxygen inside the hyperbaric chamber. Three normal, healthy human volunteers, two men and one woman with an average age of 53 $\pm$ 3 years, also underwent two hour exposures to hyperoxia.

Citrate anti-coagulated blood (16 ml) was centrifuged through Histopaque 1077 (Sigma) at 400*g* for 30 minutes to isolate leukocytes, and cells were washed in PBS. Where indicated, isolated leukocytes underwent further purification to obtain CD34+ and CD34- cells using paramagnetic polystyrene beads coated with antibody to CD34 (Dynal Biotech, Lake Success, NY). Isolation was carried out exactly as recommended by the manufacturer except that while cells were attached to the beads they were washed only twice, not three times. Normally, the bead selection system achieves 90 % purity for CD34-expressing cells, but recovers only ~ 50% of all CD34+ cells from a cell suspension. Our goal was to assess the growth potential of the CD34+ and CD34- cells separately. With our modified separation method the aspirated cells that did not attach to the CD34 antibody coated beads contained only 1.4 $\pm$ 0.4 % (SE, n=9) of the CD34-expressing cells in the total monocyte population, and the recovered cells detached from the beads were 75 $\pm$ 4 % pure. That is ~ 25 % of the monocytes used in the "CD34+" cultures did not express CD34.

For flow cytometry analysis, washed monocytes were suspended in 250 μl PBS + 0.5% bovine serum albumin (BSA). Cells were first incubated with rabbit IgG (250 μg/ml) for 5 minutes at 4^{o}C to block Fc receptors, and then incubated with a saturating concentration of R-phycoerythrin (RPE) conjugated mouse anti-human CD34 (Clone 581, a class III CD34 epitope; BD Pharmingen, San Jose, CA), fluorescein isothiocyanate (FITC) conjugated mouse anti-human CXCR4 (R&D Systems, Minneapolis, MN), and either allophycocyanin (APC)-conjugated mouse anti-human VEGF-R2 (R & D Systems) or APC-conjugated CD133 (Miltenyi Biotec, Auburn, CA) for 30 minutes at 4^{o}C. Isotype matched mouse immunoglobulin served as control. Cells were then washed with PBS, residual erythrocytes lysed by incubation in 155 mM ammonium chloride, 0.1 mM EDTA and 10 mM sodium carbonate (pH 7.2), centrifuged and resuspended in PBS. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson) at the Abramson Cancer Center Flow Cytometry Core facility. Monocytes were gated based on forward and side laser light scattering and 100,000 gated cells were analyzed for expression of cell surface markers that may be present on SPCs.

For the CFCs assays, monocytes were washed and then suspended in Metho-Cult colony assay medium (StemCell Technologies, Vancouver, BC) which contains methylcellulose, L-glutamine, fetal bovine serum, bovine serum albumin, recombinant human stem cell factor, granulocyte-monocyte colony stimulating factor, interleukin-3 (IL-3) and erythropoietin. Cultures were initiated with 1 ml of suspension/well of a 6-well Petri plate and incubated at 37^{o}C, 5% CO_2 in a fully humidified atmosphere. Non-selected monocytes were cultured at a concentration of 100,000 cells/plate, isolated CD34+ cells at 50,000 cells/plate. Colonies were apparent and counted using an inverted stage microscope at 14 days.

The phenotype of progeny cells from CFCs plates were analyzed by flow cytometry and confocal microscopy. Cells on CFCs plates were harvested by first mixing 5 ml phosphate-buffered saline (PBS) + 0.5 mM EDTA with the semi-soft Metho-Cult medium in plates and then centrifuging at 500 X G for 5 minutes. The cell pellet was washed once in PBS + 0.5 % BSA, and one aliquot of cells characterized by flow cytometry as described above. A second aliquot of cells was resuspended in growth medium and cultured in 24-well plates. Cells were suspended in 60% Dulbecco's modified Eagle's medium (low glucose; Gibco BRL, Rockford, MD), 40% MCBD-201 medium (Sigma, St. Louis, MO) and the following supplements (all purchased from Sigma) 1 X insulin-transferrin-selenium, linolenic acid-bovine serum albumin, 10^{-9} M dexamethasone, 10^{-4} M ascorbic acid-2-phosphate, 100 U penicillin and 1000 U streptomycin. After growth to confluence cells were scraped from plates, washed in PBS and spotted onto poly-lysine coated microscope slides. Cells were fixed with 1 % paraformaldehyde for 10 minutes, blocked for one hour at 4^{o}C with Tris-buffered saline (pH 8.3) containing 10 mM Tris, 250 mM NaCl, 0.3 % Tween 20 and 1 % bovine serum albumin (BSA). Cells were then covered with 50 μl 1:1000 dilution of mouse anti-human von Willebrand factor (BD Pharmingen) made up in PBS + 0.5 % BSA for 1 hour at 4^{o}C, washed twice with PBS and then counter-stained for 1 hour at 4^{o}C with a 1:2500 dilution of anti-mouse antibody conjugated to Cy3 and FITC-conjugated *Ulex europaeus* agglutinin (Sigma). Cells were examined with a BioRad Radiance 2000 attached to a Nikon TE 300 inverted stage confocal microscope that was operated with a red diode laser at 638 nm, and krypton lasers at 488 nm and 543 nm.

Mouse studies: Wild type and endothelial nitric oxide synthase knock-out (eNOS KO) mice (*Mus muscalus*) were purchased (Jackson laboratories, Bar Harbor, ME), fed a standard rodent diet and water *ad libitum*, and housed in the animal facilities of the University of Pennsylvania. Mice were exposed to HBO_2 for 90 minutes following our published protocol (27, 28). In select studies, wild type mice were pre-treated with intraperitoneal L-nitroarginine methyl ester (L-NAME) 40 mg/kg at 2 hours prior to pressurization. Blood was obtained from anesthetized mice [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)] by aortic puncture and bone marrow was harvested by clipping the ends off a femur and flushing the marrow cavity with 1 ml PBS. Leukocytes were isolated in a procedure essentially the same as that described above for human cells, except that blood cells were centrifuged through Histopaque 1083 (Sigma). Antibody staining of cell surface markers was performed as described above using FITC-conjugated rat anti-mouse Sca-1 (stem cell antigen-1) and R-PE-conjugated rat anti-mouse CD34 (both from BD Pharmingen). Mouse stem cell factor was measured using the Quantikine M immunoassay kit from R & D Systems following the manufacturer's instructions.

Bone marrow $^{\cdot}NO$ level was measured by placing microelectrodes selective for $^{\cdot}NO$ into the distal femur marrow cavity. Mice were anesthetized, the femurs exposed and a 25 gauge needle used to bore a hole through cortical bone. Nafion-coated $^{\cdot}NO$ microelectrodes, fabricated from flint glass micropipettes as described in a prior publication (33), were placed within the cavity and held in place by a micromanipulator arm assembly. The mice were then placed within the hyperbaric chamber for exposure to HBO_2. In selected studies, while breathing just air and

not HBO_2, mice received an intraperitoneal dose of sodium nitroprusside (4 to 8 mg/kg) to assess whether this manipulation would alter bone marrow $^{\cdot}NO$ concentration and mobilize SPCs.

Statistics: Statistical analysis of human stem cell numbers was carried out by repeated measures ANOVA followed by the Dunnett test (SigmaSTAT, Jandel Scientific). CFCs before and after hyperoxia were analyzed by t-test, and mouse stem cell mobilization were analyzed by ANOVA followed by Dunn's test. The level of significance was taken as $P< 0.05$ and results are expressed as mean$\pm$SE.

RESULTS

SPCs mobilization in humans: Blood from patients was obtained before and after the first, 10th and 20th hyperbaric treatments for ORN prophylaxis (the standard pre-operative course of therapy is 20 treatments). Blood leukocytes were harvested and analyzed for the presence of SPCs based on flow cytometry and colony forming cells (CFCs).

Results from flow cytometry indicated that there was a range of responses to HBO_2 and to exhibit this, results from three different patients are shown in Figures 1-3. Figure 1 panel (a) shows a typical scatter dot plot from one cell sample. Before patients were exposed to HBO_2 very few blood cells were positive for CD34, the most commonly used cell surface marker for SPCs (20). There were also few cells that expressed the receptor for vascular endothelial growth factor-2 (VEGFR-2), the receptor for stromal derived growth factor (CXCR4), or another SPCs surface marker, CD133 (20). These markers were also rarely present on cells from the paramedic attendants inside the hyperbaric chamber, who served as controls for the effect of pressure *per se*

in this study (e.g. Figure 1 c; CD133 data not shown). A comparison of Figure 1d and e shows that the number of cells expressing CD34 was increased in blood after the 1st HBO_2 treatment. Subsequent to each HBO_2 treatment we found a small elevation in a population of cells with moderately elevated CD34 expression (exhibiting intensity at between 10 and ~50) and another population with higher intensity of ~ 100 to 1000. Figure 1(f versus g) dot plots show the pattern of CD34 and VEGFR-2 expression for gated cells. Figure g shows a population of cells expressing both surface markers (upper right quadrant), and histograms (Figure 1, h and i) show the expression of VEGFR-2 on cells before and after the patient's 1st HBO_2 treatment. In all 26 patients, we found the majority of high intensity CD34+ cells also expressed VEGFR-2 at an intensity between 10 and 100.

Figure 2 exhibits responses in a patient before and after his 10th HBO_2 treatment. Cell expression of CD34 was elevated prior to the 10th treatment, and this will be discussed further below (see Figure 4). The CD34+ population in this patient exhibited somewhat lower surface expression (intensity ~100) than the patients shown in Figures 1 and 3, something we observed in a total of three patients. Circulating endothelial cells express CD34 and they may express VEGFR-2, so to more carefully discern whether HBO_2 mobilized SPCs we also probed cells for expression of CD133 and CXCR4. CD133 is not expressed by endothelial cells and CXCR4 is expressed on a subset of SPCs (5, 13, 17, 20). A population of cells expressing both CD34 and CD133 can be seen in Figure 2, panel (a) and (d) (upper right quadrant pre- and post-treatment 10). Histograms for CD34 and CD133 expression on circulating cells are also shown Figure 2. Panels in Figure 3 show responses in a third patient pre- and post- her 20th HBO_2 treatment.

We defined CD34+ cells as having fluorescence intensity above 10. As shown in Figure 4, there were persistent elevations in the circulating CD34+ populations subsequent to the first HBO_2 treatment. However, the number of leukocytes in peripheral blood was not significantly different pre/post HBO_2 (6.8 + 0.3 x $10^3/\mu l$; 27% mononuclear pre-exposure and 6.7 + 0.8 x $10^3/\mu l$; 28% mononuclear post-exposure) consistent with our previous observations (35). The fraction CD34+ cells in the gated population was 0.20+0.05% (SE) prior to the first HBO_2 treatment and 1.58+0.27 % after the 20th HBO_2 treatment, an eight-fold elevation. SPCs mobilization was due to exposure to hyperoxia, and not just pressure, because no augmentation of circulating CD34+ cells was observed in three paramedic medical attendants who assisted patients inside the hyperbaric chamber (who breathe air, not pure oxygen, while at 2.0 ATA). Figure 1, panel c shows a cell sample obtained after one paramedic underwent pressurization, and the CD34+ population looked similar to that shown in Figure 1, panels (d) and (f).

The number of CD34-expressing cells increased significantly between the first and tenth treatment. There was a trend toward a further increase (not significant) between the tenth and twentieth. While the numbers of CD34+ cells were not significantly different before versus after the 10th and 20th treatments, by the 20th treatment the subset of CD34+ cells that also expressed CXCR4 was significantly higher compared with the dually-positive population before HBO_2 started. These results are shown in Figure 5.

Circulating SPCs were also measured in three healthy human subjects prior to, and after a single 2 hour exposure to either 1 or 2 ATA O_2. There was no significant alteration in circulating SPCs due to exposure to 1 ATA O_2 (data not shown), but we found a 3-fold increase due to

exposure to 2 ATA O_2, a significantly more robust response to a single HBO_2 exposure than observed in the patient population described in Figure 4. Prior to exposure to 2.0 ATA O_2 the mean fraction of CD34+ cells was 0.20 $\pm$ 0.02% and subsequent to hyperoxia at 2 ATA, 0.67 $\pm$ 0.03% ($p<0.05$).

An alternative approach to assess SPCs was to determine the number of colony forming cells (CFCs) in peripheral blood. As shown in Figure 6, CFCs were significantly increased in response to each exposure to HBO_2. Of note, we did not find elevations in CFCs prior to the 10th and 20th treatments, although the numbers of CD34+ cells were elevated (Figure 4). As these trials were conducted in unselected monocytes, a series of trials was conducted after CD34-expressing monocytes were separated using paramagnetic beads coated with antibody to CD34 (see Methods). This procedure was carried out on cells from 9 patients prior to, and after their 20th HBO_2 treatment. We anticipated better growth in the enriched population, so cells were plated at a reduced density, 50,000 per plate, versus the 100,000/plate as in Figure 6. Prior to treatment there were 12$\pm$1 colonies/plate, and after HBO_2 23$\pm$2 colonies grew ($p<0.05$); whereas in the CD34-negative fraction, 11$\pm$ 1 colonies/plate grew prior to treatment and 11$\pm$1 (NS) grew after HBO_2.

The phenotype of progeny cells from a total of 14 patients were analyzed by flow cytometry. Cells were harvested and expression of CXCR4 and VEGFR2 was assessed. Figure 7 shows a typical expression pattern, and we could identified no discernible difference whether cells were cultured after the 1st, 10th or 20th HBO_2 treatment. Progeny cells were also sub-cultured and examined by confocal microscopy. Approximately 10% of cells heavily expressed

von Willebrand factor and stained positive for *Ulex europaeus* lectin, suggesting that a subset of the mobilized SPCs are endothelial progenitors.

Studies in mice: SPCs in peripheral blood of mice were assessed as cells that co-expressed CD34 and Sca-1. In preliminary trials, we found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA O_2. Exposure to 100 % O_2 at ambient pressure, and exposure to a pressure control, 2.8 ATA pressure using a gas containing 7.5 % O_2 (so that O_2 partial pressure was the same as ambient air, 0.21 ATA O_2) did not stimulate SPCs mobilization (Figure 8). If leukocytes were harvested immediately after the HBO_2 exposure, there was a significant increase in CD34+/Sca-1+ cells (Figure 8).

There is precedence for rapid mobilization of stem cells from bone marrow, but most emigration is believed to occur after a period of cell proliferation within the marrow niche (16, 22). We found that the number of circulating SPCs peaked at 16 hours after mice were exposed to 2.8 ATA O_2 and if mice were exposed to 2.8 ATA O_2 for 90 minutes on two successive days, the number increased even more (Figure 8). There was no additional increase in peripheral blood SPCs if mice were exposed to more than two HBO_2 treatments. The leukocyte count in peripheral blood and bone marrow did not increase in response to HBO_2, but there was a significant elevation in CFCs in both blood and bone marrow (Table 1). Soluble kit ligand (stem cell factor, SCF) was significantly elevated in peripheral blood of HBO_2-exposed mice (Table 1).

A series of studies was carried out to evaluate whether SPCs mobilization was a $^{\cdot}NO$-mediated response. We found that SPCs were not mobilized in a group of eNOS knock-out mice.

Air-exposed eNOS knock-out mice exhibited more Sca-1/CD34 dual-positive cells in the gated cell population than did wild type mice, 0.27 + 0.05 (n=4) %, but there was no evidence of stem cell mobilization in response to HBO_2. The cell level 16 hours after knock-out mice were exposed to 2.8 ATA O_2 for 90 minutes was 0.26 + 0.08 (n=5) %. We also found that if wild type mice were injected before HBO_2 with the non-specific nitric oxide synthase inhibitor, L-nitroarginine methyl ester (L-NAME), SPCs mobilization did not occur following any of the three different HBO_2-exposure protocols (Figure 8). Peripheral blood SCF was not elevated in L-NAME treated, HBO_2-exposed (Table 1).

Given that $^{\cdot}NO$ appears to be involved with SPCs mobilization, we examined whether administration of a $^{\cdot}NO$-donating agent might have an effect similar to HBO_2. We first examined the alterations in bone marrow $^{\cdot}NO$ concentrations that resulted from intraperitoneal administration of sodium nitroprusside (SNP). We started with a dose of 4 mg/kg, which is anticipated to reduce blood pressure by ~ 25% for 1 hour (31). Figure 9 shows a typical response, and also the mean response from three different trials. The $^{\cdot}NO$ concentration reached a peak of 39 $\pm$ 10 nM, and returned to baseline by 15 minutes after SNP injection. Also shown in Figure 9 is a typical response to HBO_2, which is consistent with results reported in another publication[1]. HBO_2 caused a rapid elevation in bone marrow $^{\cdot}NO$ that reached a plateau of 1008 $\pm$ 255 nM (n=3), and this level persisted for the duration of the hyperoxic exposure. With the nominal effect of SNP at 4 mg/kg, we carried out four trials using 8 mg/kg, which is approximately three-quarters of the LD_{50} for mice (39). Bone marrow $^{\cdot}NO$ was elevated by 38 $\pm$ 12 nM. We also examined circulating SPCs in mice 90 minutes after SNP administration and

found the gated cell Sca-1/CD34 dual-positive population to be 0.11 $\pm$ 0.02 %, insignificantly different from control.

DISCUSSION

The results from this study demonstrate that exposure to HBO_2 will cause rapid mobilization of SPCs in humans and the number of SPCs remain elevated in blood over the course of 20 HBO_2 treatments. Based on the responses in normal human controls, it appears that previous exposure to radiation diminishes the response to one HBO_2 treatment. Radiotherapy is known to reduce the mobilization that occurs in response to chemotherapeutic agents and growth factors (19, 25).

Studies in mice indicate that HBO_2 stimulates SPCs mobilization, although the dose-response may differ from that observed in humans. We did not systematically examine the time-course or dose-response for SPCs mobilization by HBO_2 in humans. A typical pattern for scientific/medical discovery is to carry out studies in model systems, and then verify their occurrence in human beings. In our study, we made our initial observations of SPCs mobilization by HBO_2 in humans, and then investigated the responses in animals to elucidate the mechanism. In fact, had we started our work with animals, we may not have made our discovery because the HBO_2 protocol typically used (2.0 ATA O_2 for 1-2 hours) causes only a rather nominal effect in mice. We found that the most effective pressure for increasing circulating SPCs in mice was 2.8 ATA O_2.

Observations with eNOS knock-out mice and the inhibitory effect of L-NAME in wild-type mice indicate that HBO_2 mobilized SPCs by a $^{\cdot}NO$-dependent mechanism. HBO_2 elevates SCF in peripheral blood, and this too is inhibited by L-NAME. These findings are consistent with published work showing that stimulation of bone marrow $^{\cdot}NO$ synthase will activate metalloproteinase-9 to cleave SCF from its membrane linkage, thus allowing for SCF-mediated SPCs mobilization (1, 8). HBO_2 causes a significant elevation in bone marrow $^{\cdot}NO$ concentration that cannot be replicated with infusion of SNP.

In patients, there was a significant increase in numbers of CD34+ cells between the first and 10th hyperbaric treatment. In mice, we found two treatments yielded significantly greater mobilization that one, but no further increase occurred beyond two treatments. The difference between the human and murine responses is not clear. It may be related to the apparently poorer response in patients exposed to radiotherapy versus normal controls. We did not expose the human volunteers to more than one treatment in this trial, so we do not know the optimal protocol in normal, healthy humans. An alternative possibility is that there may be differences between mice and humans in bone marrow $^{\cdot}NO$ synthesis in response to HBO_2.

There was a discrepancy between the number of CD34-expressing cells and the CFCs observed prior to the 10th and 20th HBO_2 treatments. Results from CFCs experiments conducted with purified monocyte preparations expressing CD34, and those that do not express CD34, indicate that it was the CD34+ cell population that was responsible for the increase in CFCs in response to HBO_2. It is not clear why CFCs were not elevated over the initial colony count with cells obtained prior to the 10th and 20th treatments. The improved growth subsequent to HBO_2

may relate to a small fraction of cells liberated in close proximity to HBO_2 that exhibit improved growth potential. CD34+ cells mobilized by chemotherapeutic agents and growth factors are reported to exhibit more robust growth potential than older SPCs in the circulation. Cells obtained from patients after they have undergone treatment to mobilize SPCs have twice the plating efficiency (37). Alternatively, we have not ruled out the possibility that recent exposure to HBO_2 may have an anti-apototic or pro-proliferative effect on SPCs.

Progeny cells from the CFC plates express CXCR4 and VEGFR2. As CXCR4 is required for progenitor cell homing to sites of injury/ischemia, and VEGFR2 is present on endothelial progenitors, these findings suggest that some cells mobilized by HBO_2 may function as endothelial progenitors (4, 17). This is also supported by our confocal microscopy findings. In a murine ischemic wound model we have found that HBO_2 stimulates SPCs homing to ischemic wounds, improves vasculogenesis and improves healing.[1]

Our study provides new insight into a possible mechanism for HBO_2 therapy. HBO_2 will stimulate neovascularization in humans and in animal models, although mechanisms are poorly understood (15, 24). Others have shown that HBO_2 augments growth factor synthesis (11, 14, 26). If growth factors were elevated in peripheral wounds and sites exposed to radiation, these factors would attract mobilized SPCs to home to the affected areas, where vasculogenesis could occur.

New roles for mobilized SPCs, and also elevations of SCF, are being examined in relation a number of disorders and clinical interventions (10, 12, 21, 27, 29, 32). A population of

CD34+/CD133+ cells have been shown to be pluripotent, capable of repopulating bone marrow in irradiated mice and forming dendritic progenitors (5). These studies offer impetus for further exploration with HBO_2, given its high degree of safety versus current methods of SPCs mobilization (6, 18, 38). Aural barotrauma occurs in a small number of patients, and rare occurrences of biochemical O_2 toxicity to eyes, lungs, and the CNS are virtually always reversible (6, 18, 38). An additional area where SPCs mobilization is important is the field of bone marrow transplantation (36). As mobilization of SPCs can be variable in response to chemotherapy, there may be a potential for augmenting the success of this procedure with concomitant HBO_2. This issue requires additional investigation.

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FIGURE LEGENDS

FIGURE 1: Flow cytometry analysis of human leukocytes. Data from a pressure-control subject (paramedic) and a patient before and after their first HBO_2 treatment. Panel a shows a typical forward and side-scatter dot plot and the black circle indicates the gated cell population analyzed for cell surface markers. Panel b shows a gated sampling of cells incubated with isotype-matched control mouse antibodies. Panel c shows a dot plot for cells from a paramedic, pressure control individual stained for VEGFR-2 and CXCR4. Panels d through i show data obtained from 100,000 gated cells from a patient stained for both CD34 and VEGFR-2. The second row of plots (panels d, f, and h) exhibit expression patterns for cells pre-HBO_2 and the third row of plots (panels e, g, i) show the gated cell population post-HBO_2 treatment.

FIGURE 2: Flow cytometry analysis of 100,000 human leukocytes gated as shown in Figure 1 that were stained for CD34 and CD133. Data are from a patient before and after their tenth HBO_2 treatment. Panels a through c are results obtained prior to the 10th treatment, Panels d through f are results after the 10th HBO_2 treatment.

FIGURE 3: Flow cytometry analysis of 100,000 human leukocytes gated as shown in Figure 1 that were stained for CD34 and CD133. Data are from a patient before and after their twentieth HBO_2 treatment. Panels a through c are results obtained prior to the 20th treatment, Panels d through f are results after the 20th HBO_2 treatment.

FIGURE 4: Mean CD34+ population in blood of humans before and after HBO_2 treatments. Data are the fraction of CD34+ cells within the gated population using leukocytes obtained from 26 patients before and after their 1st, 10th and 20th HBO_2 treatment. * Repeated measures one way analysis of variance, $p<0.05$ versus the pre-HBO_2 first treatment value.

FIGURE 5: Proportion of circulating CD34-expressing cells that also express CXCR4 before and after the 1st, 10th and 20th HBO_2 treatments (n=26 patients).

FIGURE 6: Colony forming cells in blood of humans before and after HBO_2 treatments. Data are the colonies counted after a 14 day incubation (all colonies had a myeloid appearance). *t-test performed on each data set pre/post-1st treatment, p=0.036; pre/post-10th treatment, p=0.041; pre/post-20th treatment, p=0.049.

FIGURE 7: Flow cytometry scatter-plot of progeny cells obtained from CFCs plates from one patient after their 10th HBO_2 treatment.

FIGURE 8: Mean CD 34+/Sca-1+ cells in blood from mice undergoing HBO_2. Control- mice not exposed to pressure or hyperoxia; L-NAME/Air- mice receiving 40 mg/kg ip L-NAME 3.5 hours before cells were harvested; 2.8 ATA pressure- mice were exposed to 2.8 ATA pressure using a gas containing only 7.5 % O_2 (0.21 ATA O_2) for 90 minutes before cells were harvested; 1 ATA O_2-mice that were exposed to 100 % O_2 at ambient pressure for 90 minutes before cells were harvested; 2.8 ATA O_2- mice were exposed to 2.8 ATA O_2 for 90 minutes and cells were harvested immediately (within 30-90 minutes) after depressurization; L-NAME/2.8 ATA O_2-

mice received 40 mg/kg ip L-NAME 2 hours before a 90 minute exposure to 2.8 ATA O_2, and then cells were harvested (in 30-90 minutes); 16 hours post-2.8 ATA O_2- mice were exposed for 90 minutes to 2.8 ATA O_2 and then left to breathe air for 16 hours before cells were harvested; L-NAME/16 hours post-2.8 ATA O_2- mice received 40 mg/kg ip L-NAME 2 hours before a 90 minute exposure to 2.8 ATA O_2, and then cells were harvested 16 hours after depressurization; 16 hours post-two exposures to 2.8 ATA O_2- mice received two 90 minute exposures to 2.8 ATA O_2 separated by 24 hours, and cells were harvested 16 hours after the second HBO_2 treatment; L-NAME/16 hours post-two exposures to 2.8 ATA O_2- mice received 40 mg/kg L-NAME ip 2 hours prior to each HBO_2 treatment, and cells were harvested 16 hours after the second HBO_2 exposure. All data show mean $\pm$ SE, and n=the number of mice in each sample. * One way analysis of variance, $p<0.05$ versus the control group.

FIGURE 9: $^{\cdot}NO$ concentration in mouse bone marrow. Panel (a) shows an example of the elevation in $^{\cdot}NO$ following 4 mg/kg sodium nitroprusside IP. Once the $^{\cdot}NO$ level returned to baseline, the mouse was pressurized to 2.8 ATA O_2, and the $^{\cdot}NO$ level found to increase markedly. A very similar pattern of responses was observed in three separate trials. Panel (b) shows the average (+ SE) elevation in $^{\cdot}NO$ observed in response to 4 mg/kg sodium nitroprusside.

Table 1. Data from mice.

	CONTROL	2.8 ATA O_2
Blood monocytes/μl	2,644 ± 306	2,103 ± 297
Marrow leukocytes/tibia	3.2±2.2 X 10^7	2.9±2.7 X 10^7
CFCs/50,000 blood leukocytes	2.6 ± 0.3	4.8 ± 1.4 *
CFCs/50,000 marrow leukocytes	17.0 ± 1.2	26.2 ± 1.5 *
Plasma SCF (pg/ml)	42.6 ± 2.8	59.5 ± 0.8 *
Plasma SCF (pg/ml) if mice pre-treated with L-NAME	39.4 ± 1.9	42.4 ± 2.5

Data are mean ± SE for studies conducted with air-breathing mice (control) and mice killed 16 hours after one HBO_2 treatment (6 mice in each group). *t-test, p=0.019 for blood CFCs data; p=0.001 for marrow CFCs data; p=0.020 for plasma stem cell factor (SCF) data.

FIGURE 1

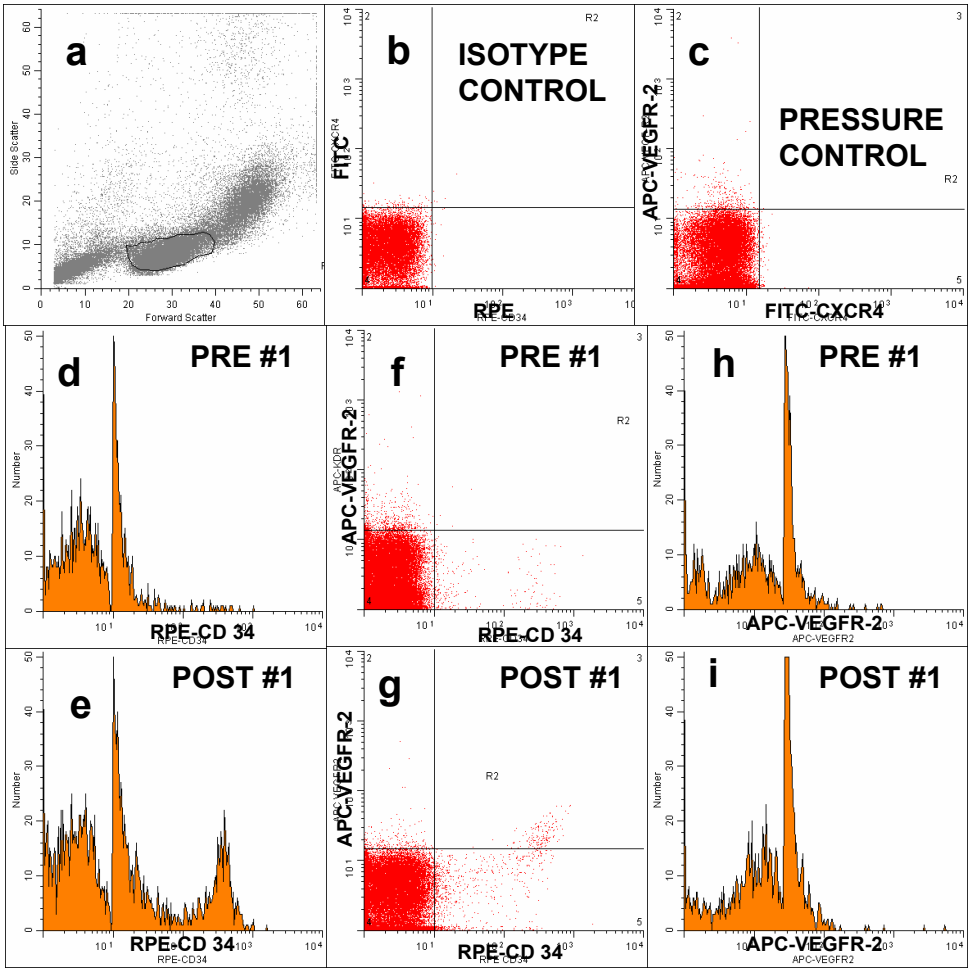
a
Side Scatter
Forward Scatter
b
ISOTYPE CONTROL
FITC
RPE
c
PRESSURE CONTROL
APC-VEGFR-2
FITC-CXCR4
d
PRE #1
Number
RPE-CD 34
f
PRE #1
APC-VEGFR-2
RPE-CD 34
h
PRE #1
Number
APC-VEGFR-2
e
POST #1
Number
RPE-CD 34
g
POST #1
APC-VEGFR-2
RPE-CD 34
i
POST #1
Number
APC-VEGFR-2

FIGURE 2

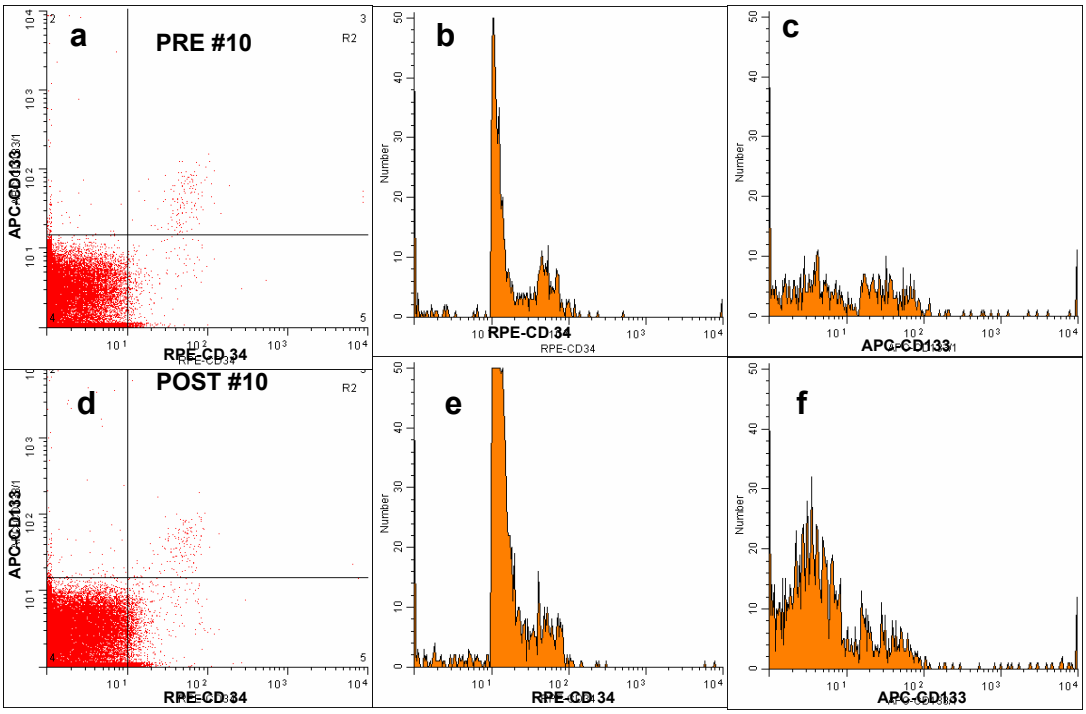
a
PRE #10
R2
APC-CD133
RPE-CD34
b
Number
RPE-CD34
c
Number
APC-CD133
d
POST #10
R2
APC-CD133
RPE-CD34
e
Number
RPE-CD34
f
Number
APC-CD133

FIGURE 3

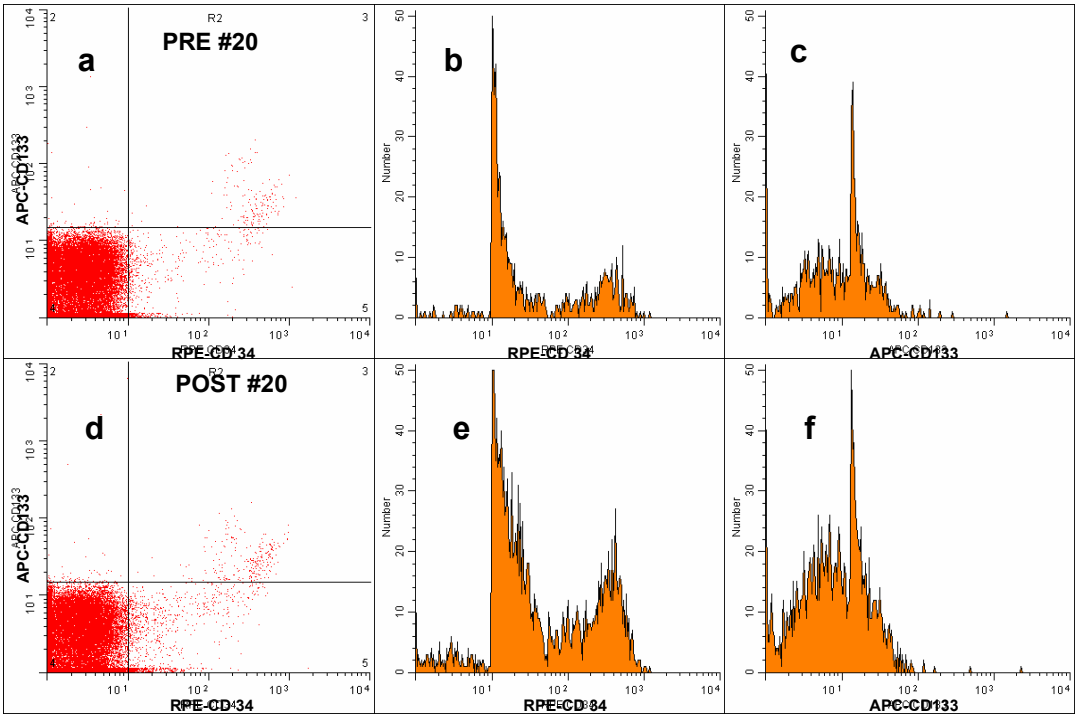
a
PRE #20
R2
APC-CD133
RPE-CD34
b
Number
RPE-CD34
c
Number
APC-CD133
d
POST #20
R2
APC-CD133
RPE-CD34
e
Number
RPE-CD34
f
Number
APC-CD133

FIGURE 4

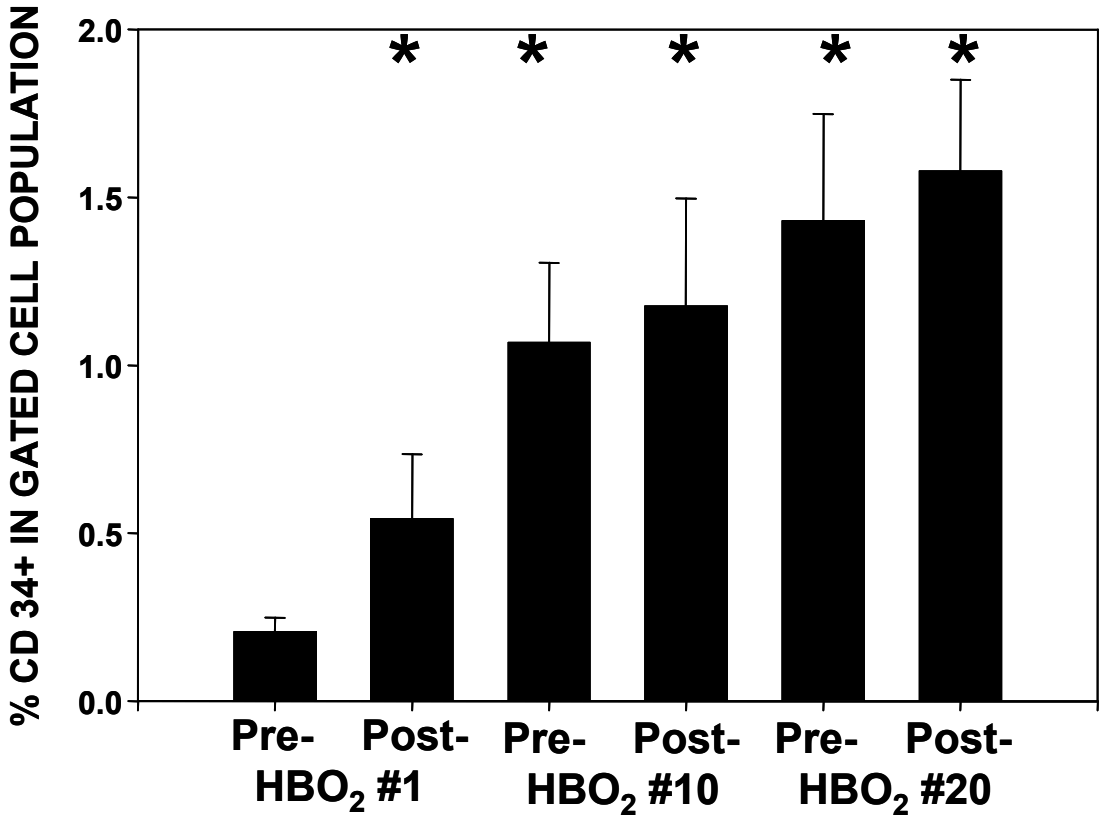
% CD 34+ IN GATED CELL POPULATION
2.0
1.5
1.0
0.5
0.0
*
*
*
*
*
Pre-HBO2 #1
Post-HBO2 #1
Pre-HBO2 #10
Post-HBO2 #10
Pre-HBO2 #20
Post-HBO2 #20

FIGURE 5

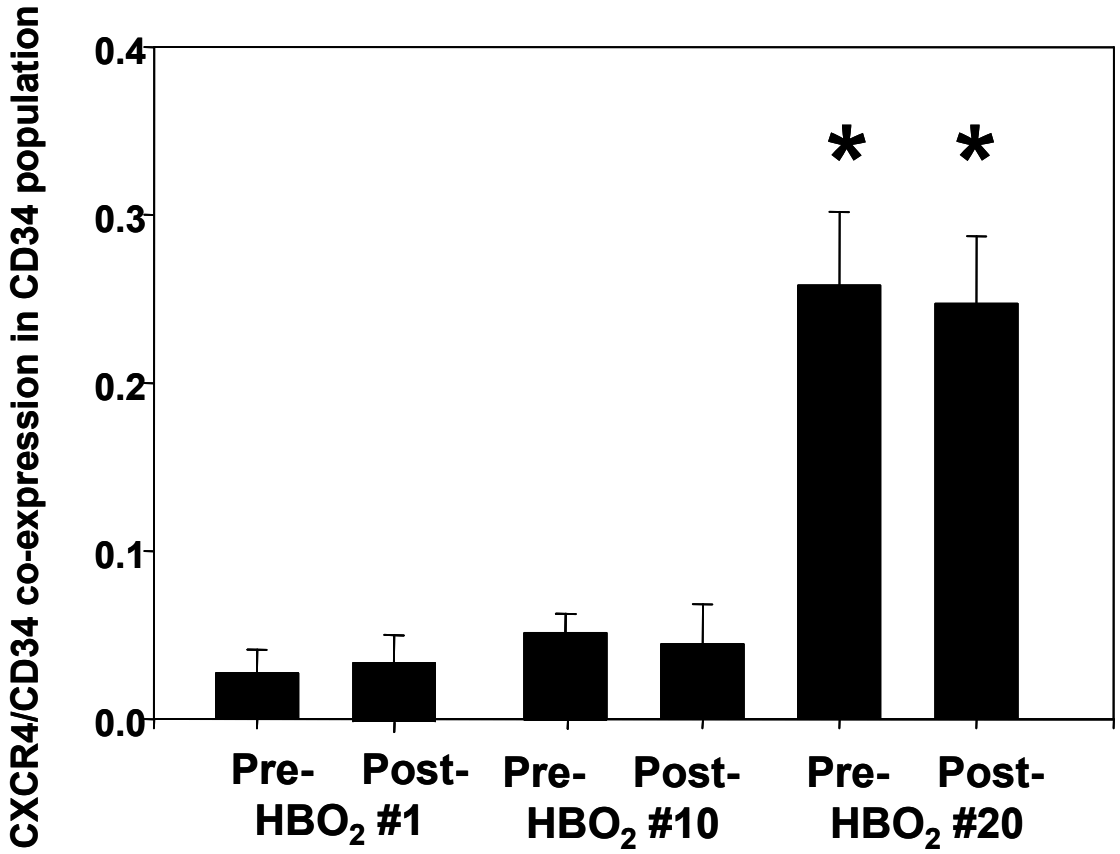
CXCR4/CD34 co-expression in CD34 population
0.4
0.3
0.2
0.1
0.0
*
*
Pre-
Post-
Pre-
Post-
Pre-
Post-
HBO2 #1
HBO2 #10
HBO2 #20

FIGURE 6

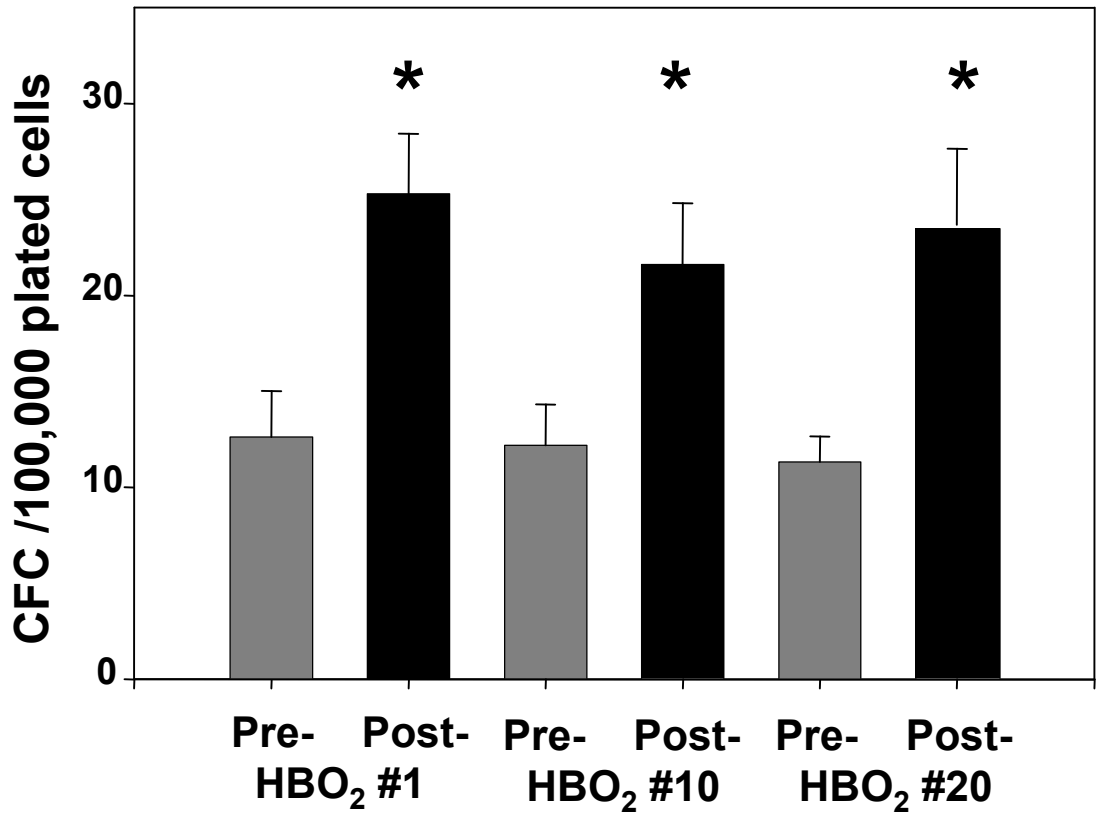
CFC /100,000 plated cells
30
20
10
0
*
*
*
Pre-HBO2 #1
Post-HBO2 #1
Pre-HBO2 #10
Post-HBO2 #10
Pre-HBO2 #20
Post-HBO2 #20

FIGURE 7

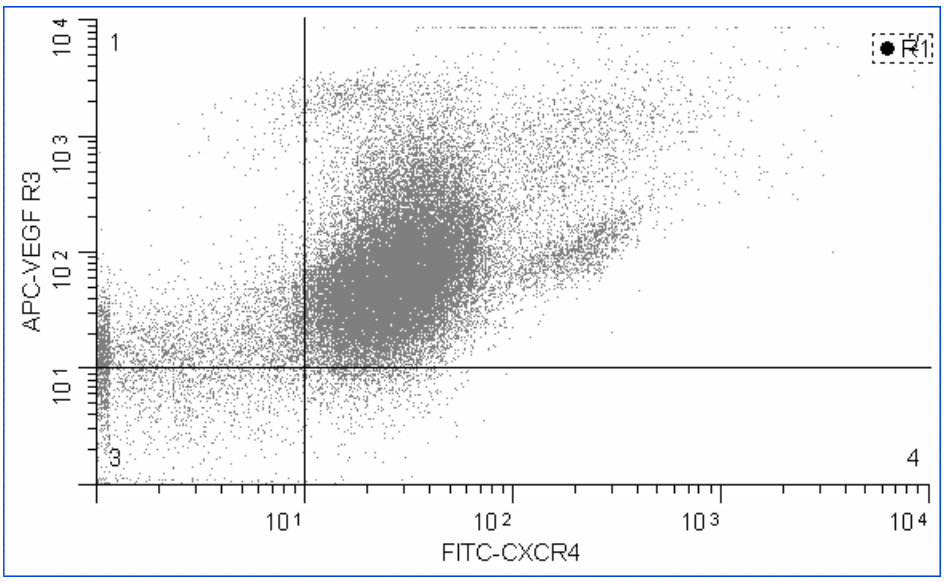

FIGURE 8

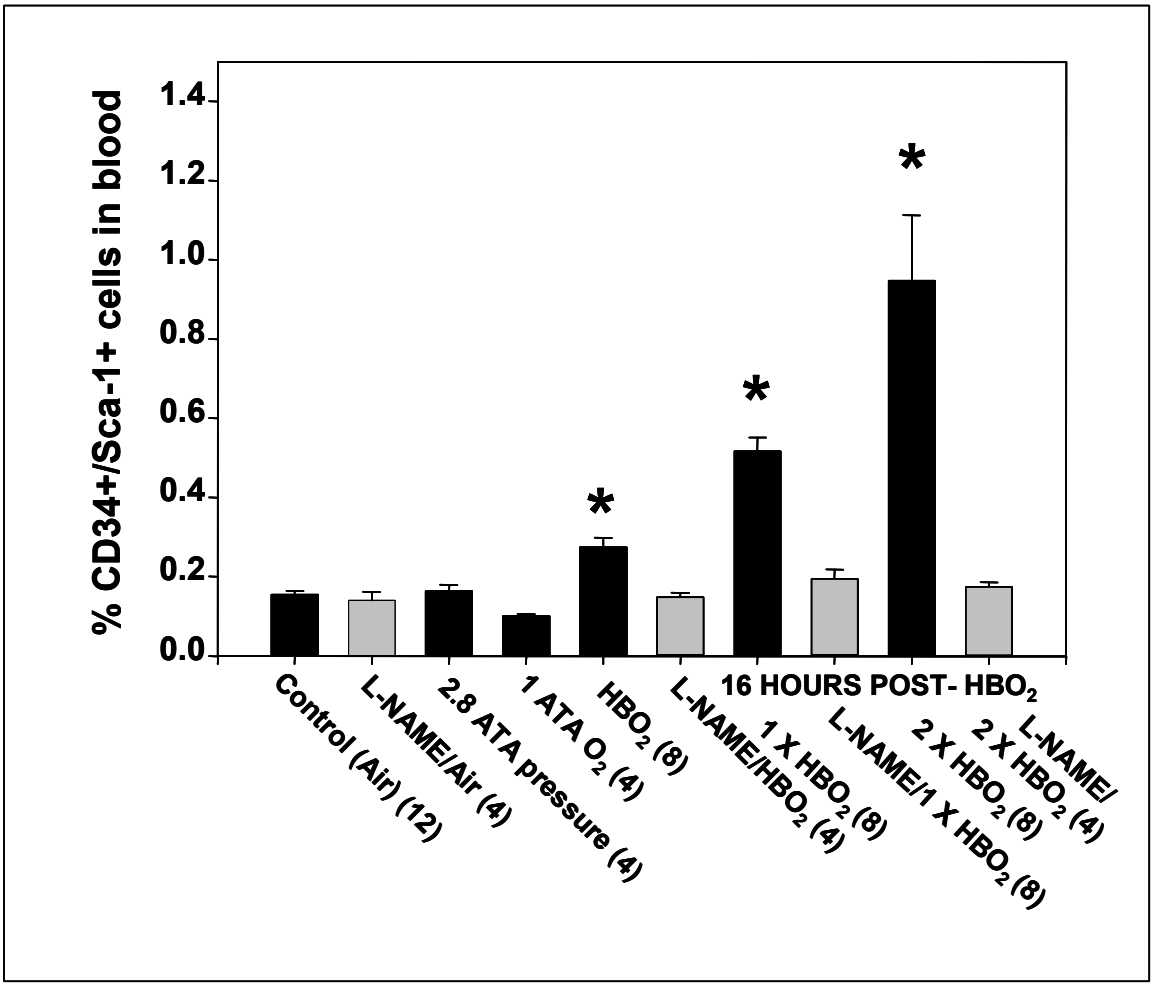
% CD34+/Sca-1+ cells in blood
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
16 HOURS POST- HBO2
Control (Air) (12)
L-NAME/Air (4)
2.8 ATA pressure (4)
1 ATA O2 (4)
HBO2 (8)
L-NAME/HBO2 (4)
1 X HBO2 (8)
L-NAME/1 X HBO2 (8)
2 X HBO2 (8)
2 X HBO2 (4)
L-NAME/

FIGURE 9