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## CD34+/CD45-dim stem cell mobilization by hyperbaric oxygen – changes with oxygen dosage

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### Abstract

Because hyperbaric oxygen treatment mobilizes bone marrow derived-stem/progenitor cells by a free radical mediated mechanism, we hypothesized that there may be differences in mobilization efficiency based on exposure to different oxygen partial pressures. Blood from twenty consecutive patients was obtained before and after the 1<sup>st</sup>, 10<sup>th</sup> and 20<sup>th</sup> treatment at two clinical centers using protocols involving exposures to oxygen at either 2.0 or 2.5 atmospheres absolute (ATA). Post-treatment values of CD34+, CD45-dim leukocytes were always 2-fold greater than the pre-treatment values for both protocols. Values for those treated at 2.5 ATA were significantly greater than the 2.0 ATA treatment group by factors of 1.9 to 3-fold after the 10<sup>th</sup> and before and after the 20<sup>th</sup> treatments. Intracellular content of hypoxia inducible factors -1,-2, and -3, thioredoxin-1 and poly-ADP-ribose polymerase assessed in permeabilized CD34+ cells with fluorophore-conjugated antibodies were twice as high in all post- versus pre-treatment samples with no significant differences between 2.0 and 2.5 ATA protocols. We conclude that putative progenitor cell mobilization is higher with 2.5 versus 2.0 ATA treatments, and all newly mobilized cells exhibit higher concentrations of an array of regulatory proteins.

### Keywords

Vasculogenic stem cells; hyperoxia; nitric oxide synthase; CD34; hypoxia inducible factors (HIF-1,2,3a); thioredoxin-1; poly-ADP-ribose polymerase

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## 1. Introduction

Stem/progenitor cells (SPCs) capable of multipotent differentiation can be mobilized from bone marrow and adipose tissue, enter the blood stream and migrate to peripheral sites where they may facilitate recovery from injuries<sup>1-3</sup>. SPCs mobilization occurs after wounding, physical exertion and in response to a variety of chemical agents<sup>4-10</sup>. Exposure to hyperbaric oxygen (HBO<sub>2</sub>) appears to be a reliable way to mobilize SPCs in humans and also has been shown in rodents and horses<sup>11-15</sup>. Animal studies indicate that one mechanism is based on activation of nitric oxide synthase type 3 (NOS-3) in bone marrow stromal cells with subsequent liberation of stem cell factor<sup>11, 16</sup>. Separate from mobilization, HBO<sub>2</sub> improves engraftment and differentiation of several progenitor cell types in organs such as spleen, bone marrow, brain, peripheral nerve, pancreas, cartilage and heart<sup>17-23</sup>. One area of interest with circulating SPCs is identification of the sub-set having propensity to form vascular endothelium, so-called endothelial progenitor cells (EPCs)<sup>24</sup>. Quantification of mobilized EPCs is based on flow cytometric detection of cell surface proteins and phenotypic manifestations of laboratory-grown clones<sup>24, 25</sup>. Cells mobilized by HBO<sub>2</sub> exhibit many of these surface markers and when cultured, some clones show lectin binding consistent with an endothelial phenotype<sup>11, 12</sup>. Animal studies have documented that HBO<sub>2</sub>-mobilized SPCs form blood vessels *in vivo* and hasten wound healing<sup>14, 16, 26</sup>.

HBO<sub>2</sub>-mobilized SPCs have greater content of hypoxia inducible factors (HIFs) and thioredoxin-1 (Trx), which in the murine model confers improved neovascularization<sup>12, 14, 27</sup>. Subsequent to HBO<sub>2</sub> treatments of refractory wounds and diabetic patients, the number of wound margin SPCs is increased and local HIFs and Trx appear to be within these localized SPCs<sup>12, 13</sup>. This suggests that SPCs play a role in supplying factors required for wound healing. Hence, evaluating intracellular proteins may have greater importance to assess SPCs function versus *ex vivo* manipulations. Assessment of intracellular regulatory proteins of cells selected based on surface markers precludes studying *ex vivo* cell growth because of need to permeabilize the cell membranes.

HBO<sub>2</sub> treatment involves breathing 100 percent O<sub>2</sub> at 2 to 3 atmospheres absolute (ATA) pressure for 1.5 to 2 hours once or twice daily. HBO<sub>2</sub> has been shown to improve refractory diabetic wounds and delayed radiation injuries in randomized trials and use is supported by independent evidence-based reviews<sup>28-34</sup>. Several studies have failed to identify clinical efficacy<sup>35, 36</sup>. Notably, these studies involved exposures to 2.0 ATA or use of face masks with questionable seals thus reducing the fraction of inspired O<sub>2</sub>; whereas several prospective randomized trials documenting therapeutic benefit utilized pressures of 2.4 or 2.5 ATA in pure O<sub>2</sub>-filled chambers or using head-covering hoods<sup>34, 37</sup>. Whether clinical results may differ because of treatment protocols is unclear. The goal of this investigation was to evaluate whether mobilization of cells with surface markers considered consistent with SPCs (CD34+ and CD45-dim) and content of intracellular regulatory proteins differed between two commonly used HBO<sub>2</sub> protocols<sup>38</sup>.

## 2. Methods

### 2.1 Patient management protocols

All procedures were approved by Institutional Review Boards and patients signed informed consent. A consecutive series of patients was approached who had been referred for HBO<sub>2</sub> treatment because of complications from radiotherapy for cancer. On the basis of current standard of care, they were to receive at least 20 HBO<sub>2</sub> therapy sessions. Patient characteristics are shown in Table 1. Venous blood was collected prior to and after the 1<sup>st</sup>, 10<sup>th</sup> and 20<sup>th</sup> HBO<sub>2</sub> treatment into Cyto-Chex BCT test tubes (Streck, Inc., Omaha, NE) that contain a proprietary preservative. Samples from the same day of treatment (pre- and post-HBO<sub>2</sub>) were analyzed concurrently within 3 days of collection.

The standard Penn-based practice for delivering O<sub>2</sub> involved placement of a balloon-cushioned face mask that is normally used for continuous positive airway pressure respiratory therapy. Treatments were conducted at 2.0 ATA for 2 h daily, 6 days/week. Intermittently the fractional inspired O<sub>2</sub> content in the mask was verified to be 100%. Syracuse-based treatments were conducted in an acrylic chamber pressurized with pure O<sub>2</sub> so that no special mask was required to assure 100% O<sub>2</sub> delivery. Treatments were at 2.5 ATA for 90 minutes daily, 6 days/week.

### 2.2 Flow cytometry

CD34+ and CD45-dim cells and relative concentrations of intracellular proteins were evaluated with a 10-color FACSCanto (Becton Dickinson, San Jose, CA) using standard acquisition software following published techniques<sup>12, 14, 27</sup>. Briefly, nucleated cells were segregated from debris by DRAQ5 DNA staining and gates were based on true-negative controls according to fluorescence-minus-one analysis. Anti-actin fluorescence confirmed uniform cell permeabilization for intracellular protein analysis. Fluorescence/cell was determined and used to compare pre- versus post-HBO<sub>2</sub> cell populations.

### 2.3 Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from the following sources: From BD Pharmingen, San Jose, CA. R-phycoerythrin (PE)-conjugated mouse anti-human CD34 (Clone 581, a class III CD34 epitope; catalogue number 555822), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45, catalogue number 5558710 and allophycocyanin (APC)-conjugated mouse anti-human poly-ADP ribose polymerase (PARP) catalogue number 558710; from R & D Systems, Minneapolis, MN, APC-conjugated anti-human hypoxia inducible factor (HIF)-1, catalogue number IC1935P; from Novus Biologicals, Littleton, CO, PE-conjugated anti-human HIF -2 (catalogue number NB100-122, FITC-conjugated anti-human HIF-3 (catalogue number NB100-2529) and anti-human Trx catalogue number EPR 6111 with secondary from Invitrogen, Grand Island, NY catalogue number T-2769.

### 2.4 Statistical analysis

Statistical analysis of stem cell numbers and quantitative changes in wound protein markers were carried out by repeated measures analysis of variance followed by the Tukey test for

multiple comparisons (SigmaStat, Jandel Scientific, San Rafael, CA). Statistical significance was taken as  $p < 0.05$ . Data sets were found to be normally distributed so results are displayed as mean  $\pm$  SE,  $n=20$  for all groups. Pre- and post-treatment comparisons were made within each type (2.0 ATA and 2.5 ATA) and between the 2.0 and 2.5 ATA treatments for each number (1<sup>st</sup>, 10<sup>th</sup> and 20<sup>th</sup>) by two-tailed t-test.

### 3. Results

#### 3.1 Circulating cells

Circulating CD34+ and CD45-dim leukocytes increased in blood from 20 consecutive patients undergoing HBO<sub>2</sub> therapy following a protocol of either 2.0 ATA or 2.5 ATA (Figure 1). There were no significant differences in age, gender or radiation dose between groups (Table 1). Following the 10<sup>th</sup> as well as before and after the 20<sup>th</sup> treatment cell counts were significantly higher with the 2.5 ATA versus the 2.0 ATA protocol. Findings were essentially the same whether normalized to volume of blood (left axis of Figure 1) or to total circulating leukocyte count (right axis) because total leukocyte counts for patients did not differ significantly over the course of the HBO<sub>2</sub> treatments (data not shown).

#### 3.2 Intracellular protein concentrations

Significant elevations of intracellular regulatory proteins were found in permeabilized CD34+ cells after the 1<sup>st</sup>, 10<sup>th</sup> and 20<sup>th</sup> treatments with either protocol (Table 2). Because of variations in fluorescence intensity due to different lots of antibody and also flow cytometer laser intensities, only differences in cell fluorescence intensity for pre- and post-HBO<sub>2</sub> samples analyzed on the same day were compared and not intensity across a 20 treatment course.

### 4. Discussion

The results demonstrate that O<sub>2</sub> partial pressure influences SPCs mobilization with repetitive treatments. Whether this is due to augmented NOS-3 activation requires additional study. SPCs mobilization in response to a variety of drugs is compromised by older age, prior radiotherapy and use of several types of chemotherapy (*e.g.* platinum compounds, alkylating agents, purine analogues and lenalidomide)<sup>39</sup>. None of these agents were being administered to patients during our study. We have reported previously that SPCs mobilization in response to a single 2.0 ATA O<sub>2</sub> exposure is the same between normal adults and those exposed to radiotherapy<sup>11</sup>. Obviously, all patients in this study received radiotherapy but there was no significant difference in radiation dosage or patient age between the 2.0 and 2.5 ATA treatment groups (Table 1).

There were no notable deviations in the pattern of SPCs mobilization among the patients despite taking a variety of medications listed in Table 1. Some of these medications are known to have positive effects on SPCs mobilization (*e.g.* short term statin use, paclitaxel, certain  $\beta$ -blockers such as nebivolol and carvedilol; while others have a negative impact on mobilization (*e.g.* bisphosphonates, long-term use of statins and trimethoprim/sulfamethoxazole)<sup>40-46</sup>. None of the medications listed in Table 1 had been started in the time frame while patients were receiving HBO<sub>2</sub> and all had been prescribed for over 2

months prior to patient enrollment. One patient in the 2.0 ATA group had HIV and one in the 2.5 ATA group had renal failure and was undergoing dialysis. HIV does not impede the efficacy of chemotherapeutic agent-mediated SPCs mobilization and renal failure may modify mobilization by some drugs but does not completely abrogate responses<sup>47–50</sup>. Whether these disorders influence HBO<sub>2</sub>-mediated mobilization will require additional study. Clearly, there are differences in mobilization mechanisms between chemotherapeutic agents and HBO<sub>2</sub>. Contrary to many of the stem cell mobilization drugs HBO<sub>2</sub> does not activate platelets or elevate leukocyte counts which can be thrombogenic<sup>13, 51–53</sup>.

Intracellular regulatory protein contents were elevated in all post-HBO<sub>2</sub> samples with no significant differences between protocols. Elevations are likely a characteristic of the bone marrow SPCs population primed for mobilization and a higher percentage is released with higher O<sub>2</sub> dose. Lower protein levels in pre-HBO<sub>2</sub> samples at the 10<sup>th</sup> and 20<sup>th</sup> treatments may reflect preferential perivascular sequestration of newly mobilized cells and/or protein degradation in cells remaining in the circulation for many hours. The difference in protein contents of newly mobilized SPCs has not been appreciated in mobilization studies involving chemotherapeutic agents. This is probably because responses to chemical agents proceed over a much longer time course.

A weakness of this investigation is that perhaps alternative or additional surface markers should be used to better characterize the mobilized cells. With regard to neovascularization potential, this is difficult to determine given the ongoing debate over EPCs characterization<sup>38</sup>. Elevated intracellular proteins of HBO<sub>2</sub>–mobilized cells suggest they may have improved propensity for growth/differentiation based on animal studies<sup>14, 27</sup>. HIF-3 and PARP were probed because they provide evidence that cells were not merely circulating endothelial cells or cells undergoing apoptosis. PARP levels would be expected to be quite low in apoptotic cells<sup>54</sup>. EPCs can be distinguished from mature CECs by determining ‘clonogenic’ proliferative capacity, but not by flow cytometric evaluation of surface markers<sup>38</sup>. Our approach for assessing intracellular markers after membrane permeabilization precludes *ex vivo* growth analysis, which is why we probed for HIF-3. In animals we have found HBO<sub>2</sub>–mobilized SPCs that form new blood vessels and hence not CECs are well endowed with HIF-3, whereas HIF-3 normally is highly tissue restricted (to thymus, lung and a lesser extent in brain, heart and kidney)<sup>14, 55</sup>. Therefore, we conclude that the cells mobilized by hyperoxia are SPCs and that treatment pressure influences mobilization efficiency. Functional consequences of this response require further study.

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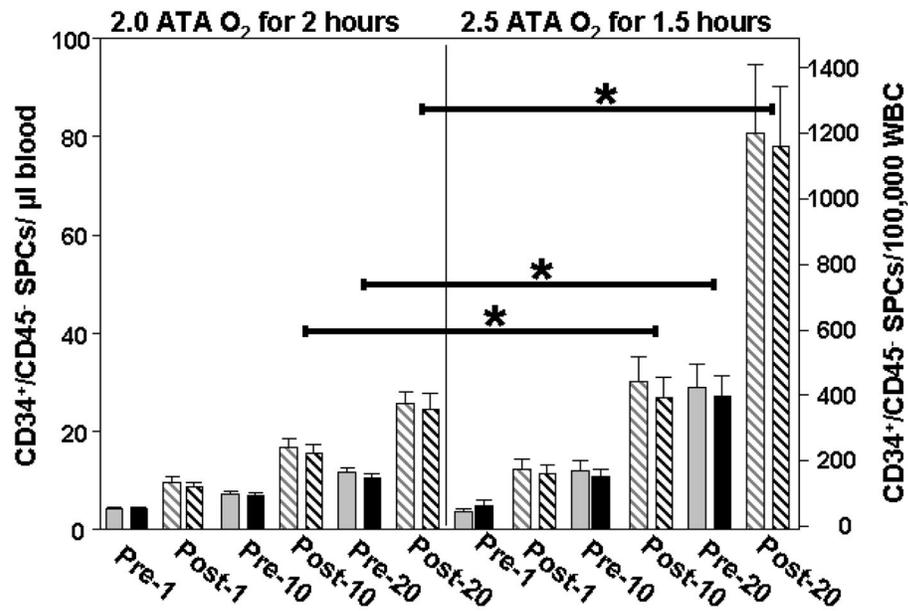
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### Highlights

1. The number of circulating CD34<sup>+</sup>-CD45<sup>-</sup> leukocytes are doubled in humans within 2 hours of exposure to oxygen at 2.0 or 2.5 atmospheres absolute (ATA).
2. Repetitive exposures to 2.5 ATA leads to a further 1.9 to 3.0-fold elevation of CD34<sup>+</sup>-CD45<sup>-</sup> cells with up to 20 treatments versus 2.0 ATA treatments.
3. Newly mobilized CD34<sup>+</sup>-CD45<sup>-</sup> leukocytes exhibit higher concentrations of an array of regulatory proteins.



**Figure 1. Leukocyte mobilization by HBO<sub>2</sub>**

The number of circulating CD34<sup>+</sup>,CD45<sup>-</sup>dim cells in blood before and after the 1<sup>st</sup>, 10<sup>th</sup> and 20<sup>th</sup> treatment of 20 patients exposed to either at 2.0 or 2.5 ATA. Data were normalized to blood volume (grey boxes quantified on the left ordinate axis) or to total circulating leukocyte count (black boxes quantified on the right ordinate axis) and are mean ± SE, \* indicates significant difference between 2.0 and 2.5 ATA groups (ANOVA). All post-HBO<sub>2</sub> values are significantly different from pre-HBO<sub>2</sub> values at each treatment time in both groups (t-test).

**Table 1**

**Patient characteristics**

Details for the Penn and Syracuse treatment centers show age/gender, cancer location, radiation dose (cGy), other health issues, tobacco and ethanol use. Penn-based patients were 62.1 ± 2.4 (mean ± SE) years old, 5 were female; Syracuse-based patients were 62.0 ± 2.5 years old (NS), 5 were female. Radiation dosage was known in 14 Penn-based patients (6409 ± 133 cGy) and 12 Syracuse-based patients (6635 ± 345, NS).

Penn#	Age/Gender	Cancer	Radiat.	Other	Medications	Tobacco	Ethanol
1	43M	Tongue	6000	HTN, HIV, COPD, Epilepsy, Asthma	Fosamprenavir, ritonavir, trimethoprim-sulfamethoxazole	None	None
2	67M	Tongue	6600	HTN, DM-2, Adrenal CA	Amlodipine, terazosin, senna, MVI	None	Occasional
3	68M	Prostate	Brachy, ND	Depression	Morphine, oxycodone, docusate sodium, omeprazole, citalipram, cyclobenzaprine HCl, gabapentin	Quit >6 weeks	None
4	58F	Sinus	5400	HTN, DVT, Cataracts, Glaucoma	Amlodipine, warfarin, alendronate,latanoprost gts, ciprodex gts	None	None
5	39F	Cervix	ND	Depression	Paclitaxel, methadone, gabapentin, bupropion HCl fluoxetine, pentosan polysulfate, alprazolam, clonazepam	None	Occasional
6	55M	Neck	6530	HTN, CAD, CVA, Cholesterol,	Ibuprofen, ranitidine, MVI	None	None
7	63M	Larynx	ND	COPD, Hypothyroid	Gabapentin, levothyroxine	Quit >3 yrs	Occasional
8	68M	Tongue	ND	Lymphoma, Hypothyroid	Ramapril, levothyroxine	None	Occasional
9	57M	Tonsil	6300	None	Pregabalin, oxycodone, lansoprazole, MVI, glycopyrrolate	Quit >3 yrs	Occasional
10	67M	Tongue	7000	GERD	Esomepra zole	None	None
11	48M	Tonsil	6300	Asthma	Albuterol, gabapentin, glycopyrrolate, oxycodone	Quit >5 yrs	None
12	65F	Tongue	5580	Hypothyroid, Cataracts	Levothyroxine	None	Occasional
13	76M	Prostate	ND	HTN, Cholesterol	Metoprolol, amlodipine, pravastatin	Quit >25 yrs	Occasional
14	79M	Tongue	6820	HTN, DM-2, COPD	Metoprolol, losartan, HCTZ, irbesartan, chlorpheniramine-hydrocodone syrup	Quit >15 yrs	Occasional
15	68F	Tongue	7000	HTN, GERD, Cholesterol, CAD	nebivolol, clopidogrel, rosuvastatin, metoclopramide	Quit >3 yrs	Occasional
16	56M	Tongue	ND	Cholesterol, CAD	Pentoxifylline, rosuvastatin, oxycodone	None	Occasional
17	78M	Prostate	Brachy, ND	HTN, Crohn's	Atenolol	None	None
18	56M	Neck	6600	HTN, Migraine, Cholesterol, Gout	Ezetimibe, pitavastatin, rizatriptan, allopurinol	Quit >15 yrs	Occasional
19	70M	Tongue	7000	HTN, Hypothyroid,	Lisinopril, celecoxib, tramadol, trazodone, gabapentin, levothyroxine, MVI	None	Occasional
20	60F	Tonsil	6300	None	Pentoxifylline, oxycodone	None	Occasional

Syracuse#	Age/Gender	Cancer	Radiat.	Other	Medications	Tobacco	Ethanol
1	50M	Tonsil	6996	ITP	Prednisone, oxycodone, xanax, fentanyl, sertraline	Chew	None
2	73F	Tonsil	7000	COPD, Esoph Ca	Albuterol, fluticasone, esomeprazole, mometasone	Quit >5 yrs	None
3	53M	Mouth	7300	HTN	Morphine, oxycodone, colace, omeprazole, citalipram, cyclobenzaprine, gabapentin	Quit >6 weeks	None
4	72M	Prostate	6600	Cholesterol	Simvastatin	Quit >5 yrs	None
5	55M	Mouth	ND	AFib, Aortic valve	Coumadin, carvedilol, amlodipine, fluticasone, albuterol, acetaminophen-hydrocodone	None	None
6	61M	Mouth	ND	HTN, Esoph strictures	Bisacodyl, lisinopril, omeprazole, ranitidine, sulfamethoxazole, trazadone	Quit >8 weeks	Occas.
7	60M	Mouth	6600	None	Ibuprofen, ranitidine, MVI	None	None
8	53M	Palate	7000	Sinusitis	Pregabalin	Quit >3 yrs	Occas.
9	57M	Larynx	ND	Laryngect.	Gabapentin, cyclobenzaprine, oxycodone, nortriptyline, HCTZ	Quit >3 yrs	None
10	68M	Tongue	6400	Lung CA, COPD	Alendronate, travatan ophthalmic sol, albuterol inh, levothyroxine, fluticasone inh, ferrous sulfate	Quit >5 yrs	None
11	76F	Breast	ND	Aortic valve	Bumetanide, atorvastatin omeprazole, ASA, levothyroxine, KCl, fluticasone, naproxen, acetaminophen-hydrocodone, pseudoephedrine, Vitamin D, MVI	None	None
12	79M	Prostate	ND	HTN, Colon CA	Coumadin, amlodipine, atorvastatin, HCTZ, bicalutamide	Quit >5 yrs	None
13	57F	Larynx	6996	HTN	Lisinopril, methimazole, carvedilol	1 PPD	None
14	51M	Neck	ND	HTN, Cholesterol, Hypothyroid	Levothyroxine, bisoprolol, pantoprazole, pravachol, MVI	None	Occas.
15	46M	Mouth	ND	None	Acetaminophen	1 PPD	None
16	69M	Prostate	7740	HTN, Cholesterol, Asthma	Albuterol inh, fluticasone inh, tiotropium inh, amlodipine, rosuvastatin, acetaminophen, fluticasone	None	None
17	82F	Mouth	3000	HTN, CAD, Dialysis	Levothyroxine, isosorbide, vitamin b complex, donepezil, metoprolol, oxycodone, mesalamine, citalopram, nortriptyline, omeprazole, diphenoxylate, folate, MVI	None	None
18	69F	Tongue	ND	HTN, Cholesterol, Asthma	Albuterol inh, amlodipine, atorvastatin	None	None
19	46M	Palate	6996	Epilepsy	Lamictal, omeprazole, tramcinolone	None	None
20	62M	Tongue	6996	HTN	HCTZ	Quit >5 yrs	None

Abbreviations used are as follows: ND, not determined (not available from chart or the referring physicians); Brachy, brachytherapy; HTN, hypertension; HIV, human immunodeficiency virus infection; COPD, chronic obstructive pulmonary disease; DM-2, type 2 diabetes mellitus; Ca, cancer; DVT, deep venous thrombosis of a leg; CAD, coronary artery disease; CVA, cerebrovascular accident; GERD, gastro-esophageal reflux disease; Cholesterol, hypercholesterolemia; Esoph, esophageal; Afib, atrial fibrillation; Larengect., laryngectomy; aortic valve, history of aortic valve replacement. HCTZ, hydrochlorothiazide; MVI, multivitamin pill.

**Table 2**

Intracellular protein content (fold-elevation post- versus prior to HBO<sub>2</sub>).

Protein	Treatment #	2.0 ATA Protocol	2.5 ATA Protocol
HIF-1	1	2.35 ± 0.24	3.29 ± 0.55
	10	2.65 ± 0.21	2.67 ± 0.22
	20	2.54 ± 0.38	2.77 ± 0.26
HIF-2	1	2.33 ± 0.24	2.68 ± 0.30
	10	2.48 ± 0.15	2.54 ± 0.20
	20	2.54 ± 0.23	2.60 ± 0.21
HIF-3	1	2.27 ± 0.22	2.67 ± 0.31
	10	2.38 ± 0.24	2.29 ± 0.15
	20	2.43 ± 0.26	2.27 ± 0.15
Trx	1	2.34 ± 0.24	2.51 ± 0.26
	10	2.36 ± 0.22	2.28 ± 0.13
	20	2.44 ± 0.24	2.50 ± 0.29
PARP	1	2.36 ± 0.22	2.64 ± 0.26
	10	2.39 ± 0.22	2.42 ± 0.19
	20	2.57 ± 0.27	2.47 ± 0.22

Data show mean ± SE fold-differences in fluorescence of post-versus pre-HBO<sub>2</sub> permeabilized CD34<sup>+</sup> cells using fluorophore-conjugated antibodies to proteins shown in column 1. All post-HBO<sub>2</sub> values are significantly different from pre-HBO<sub>2</sub> and there are no significant differences between the 2.0 and 2.5 ATA protocols.