Only minor stem cell mobilization in head and neck irradiated patients treated with hyperbaric oxygen

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Abstract
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Introduction: Hyperbaric oxygen, (HBO) is used to treat several conditions including late radiation tissue injury. Previous studies have suggested that HBO mobilizes bone marrow derived stem/progenitor cells (SPC) to the peripheral blood, however possible cumulative effects were highly variable.
Methods: We have investigated a possible HBO-induced mobilization of SPCs by determining CD34+CD45dim cell numbers, as well as SPCs in general. The latter were characterized by high aldehyde dehydrogenase (ALDH) activity by use of the Aldefluor® assay. We included ten patients admitted for HBO treatment of radiation tissue injury. Six patients completed the 29–30 HBO treatment exposures. We also investigated possible HBO-induced effects on platelet activation as measured by flow cytometry and functional analyses.
Results: We found a weak and insignificant tendency toward mobilization of CD34+CD45dim cells after a single HBO exposure versus before. Additionally, we found an additive effect of 15 HBO exposures on the increase in CD34+CD45dim cells relative to the pre-1st-HBO values. These changes were significantly more than zero but less than a doubling. We could not demonstrate a significant effect of HBO on the content of Aldefluor® positive SPCs in peripheral blood. There was no significant effect on platelet activation overall. However, in patients with increased expression of activation markers at baseline, we found a decrease after one exposure although this was not reflected in functional tests.
Conclusion: We found a minor statistically significant mobilizing effect of HBO treatment on the bone marrow derived stem/progenitor cell content in peripheral blood after 15 treatments (n = 10 patients), but no effect after 30 treatments (n = 6 patients). However, because of the low number of patients we cannot confidentially prove or disprove the null hypothesis. The possibility that HBO treatment reduces the number of activated platelets could not be demonstrated nor excluded.

Introduction

Hyperbaric oxygen (HBO) is used for the treatment of a variety of conditions including late radiation tissue injury. HBO treatment has been shown to increase the number of small blood vessels in irradiated tissue. This results in increased tissue oxygen levels and improved white cell and fibroblast function, which further enhances wound healing.[1–3]

In addition, previous studies have suggested that HBO treatment induces mobilization of bone-marrow-derived stem/progenitor cells (SPCs) through, e.g., HBO-mediated oxidative stress at sites of neovascularization.[3–5] Bone marrow derived SPCs encompass a variety of progenitor cells including hematopoietic stem cells (HSCs), mesenchymal stromal cells (MSCs), and endothelial progenitor cells (EPCs).[6] Potentially, mobilization of these cells could facilitate regeneration of radiation injured tissue by EPC-induced vasculogenesis[7,8] and/or the anti-inflammatory and anti-fibrosis activity of MSCs[9] after homing of these cells to ischaemic areas. However, although three clinical studies have reported more than a doubling of circulating SPCs after one HBO exposure, the possible cumulative effect after 20 exposures has varied considerably between studies from none to more than a 20-fold increase in circulating SPCs.[4,10,11]

Determination of the mentioned cell populations can be performed by analysis of specific surface markers

characterizing each population. In addition, SPCs can collectively, regardless of the specific type, be characterized by their high activity of the cytosolic enzyme aldehyde dehydrogenase (ALDH). The SPCs therefore can be labelled by a cell-permeable fluorescent substrate (e.g., Aldefluor®) which is converted in the cytoplasma by ALDH to a charged molecule unable to leave the cell, and therefore accumulating in cells with high ALDH activity.[6]

With respect to inflammation, platelets secrete a variety of peptides and proteins in their activated state and influence the attraction of leukocytes to the endothelium.[12,13] Regarding a possible HBO-induced anti-inflammatory effect, Shaw et al., demonstrated an HBO-mediated up-regulation of relevant proteins from platelets.[14] Additionally, some studies have shown altered aggregation or numbers of platelets after HBO[15–17] while others have shown no such impact of HBO.[17,18]

In the present study, we investigated whether HBO treatment given to a group of patients with radiation induced injuries brought about a rise in peripheral blood of Aldefluor®-positive cells, representing a general SPC marker, and/or of CD34+CD45dim cells as a certain marker of HSCs and a reported marker of the much-debated EPC.[19] Moreover, we investigated whether HBO treatment induced changes in platelet activation using both flow cytometry and functional tests.

Methods

The study had an open prospective design. The study was approved by The Regional Scientific Ethics Committee of The Capital Region of Denmark and the Danish Data Protection Agency (Ethics Committee Approval Number was H-1-2010-093). Patient consent was obtained before inclusion.

PATIENTS

Ten patients received radiotherapy according to the Danish national guidelines (available from: https://www.dahanca.dk) to total doses to the tumor area of 66–68 Gy, and prophylactic nodal irradiation to a total dose of 46–50 Gy. Radiotherapy was given in 2 Gy fractions with 5 or 6 fractions per week. Each participant was referred to the hyperbaric facility because of osteoradionecrosis (ORN) or late radiation tissue injury (LRTI), including prophylactic treatment before tooth extraction. All patients with a diagnosis of mandibular/maxillary osteoradionecrosis or late radiation tissue injury were considered eligible.

Table 1 provides basic demographic information, details of the cancer diagnosis, and number of hyperbaric treatments. Table 2 provides information about patient comorbidities and medication. The exclusion criteria were: age < 18, uncontrolled hypertension, epilepsy, lack of ability to equalize pressure in the middle ear without the need to insert a drain, unmanageable claustrophobia, presence of or suspicion of pneumothorax, thoracic surgery within the last month, haemoglobin < 6 mmol·mL^{-1} (9.67 g·dL^{-1}).

HYPERBARIC OXYGEN TREATMENT

The patients were placed in a multi-place pressure chamber breathing 100% oxygen from a hood (Amron Vista, California, USA). The treatment consisted of pressurization over 5 minutes to a pressure of 243 kPa (2.4 ATA). The pressure was maintained for 90 minutes (no air breaks) followed by decompression over 5 minutes. The patients were subjected to one daily exposure five days a week for six weeks for a total number of 29–30 exposures.

BLOOD SAMPLES

Six mL of ethylenediaminetetraacetic acid (EDTA) stabilized blood was obtained before and after exposures 1, 15 and 29 or 30 to determine the absolute concentration of CD34+CD45dim cells in whole blood. Before and after exposure numbers 1 and 29 or 30, an additional 12 mL of EDTA stabilized blood was obtained for isolation of the mononuclear cell (MNC) fraction and determination of the Aldefluor® positive and CD34+CD45dim cells relative to the MNC population.

For platelet activation analyses, 3.5 mL of citrate stabilized blood and 4 mL of heparin stabilized blood was obtained before and after exposures 1 and 29 or 30.

LABORATORY METHODS

Haematological parameters

Blood cell counts, i.e. haematocrit, total white blood cells, granulocytes, monocytes and platelets were determined using a Sysmex® XE-2100D analyser (Sysmex, Kobe, Japan) according to the manufacturer's instructions.

Stem cell determination

CD34+CD45dim cell numbers were determined both by use of a single-platform on whole blood and by determining values relative to lymphocytes using MNCs obtained by lymphoprep density separation. Aldefluor® positive cells were determined only relative to MNCs. All flow cytometric measurements were done in duplicate.

For MNC separation, EDTA anticoagulated blood diluted 1:1 with RPMI 1640 (Sigma-Aldrich, Missouri) was centrifuged through Lymphoprep™ (STEMCELL Technologies, Inc., UK) 800 g for 20 min according to the manufacturer's instructions.

Aldefluor® staining with immunophenotyping of the MNC suspensions was performed using Aldefluor® staining reagents (Aldagen Inc., Durham NC, USA) and the

Table 1
Patient characteristics including demographic information, details of the cancer diagnosis, and number of hyperbaric treatments. Exp = HBO exposures; F = female; LRTI = late radiation tissue injury; M = male; ORN = osteoradionecrosis

No.	Gender /age	HBO indication	Cancer year	Localization	Exp.	Duration (days)
1	M/76	Tooth extraction	1983	Larynx	30	49
2	M/63	ORN	2008	Lingual base	29	44
3	M/63	LRTI	2010	Retromolar trigonum	24	41
4	M/47	Tooth extraction	2002	Parotid gland	30	42
5	F/81	Tooth extraction	2005	Hodgkins lymphoma	30	54
6	M/51	ORN	2011	Tonsil	30	42
7	M/63	ORN	2009	Cheek mucosa	30	42
8	F/59	ORN	2005	No information	22	42
9	F/57	LRTI	2005	Oral cavity	29	60
10	M/72	ORN	2008	Tonsil	27	43

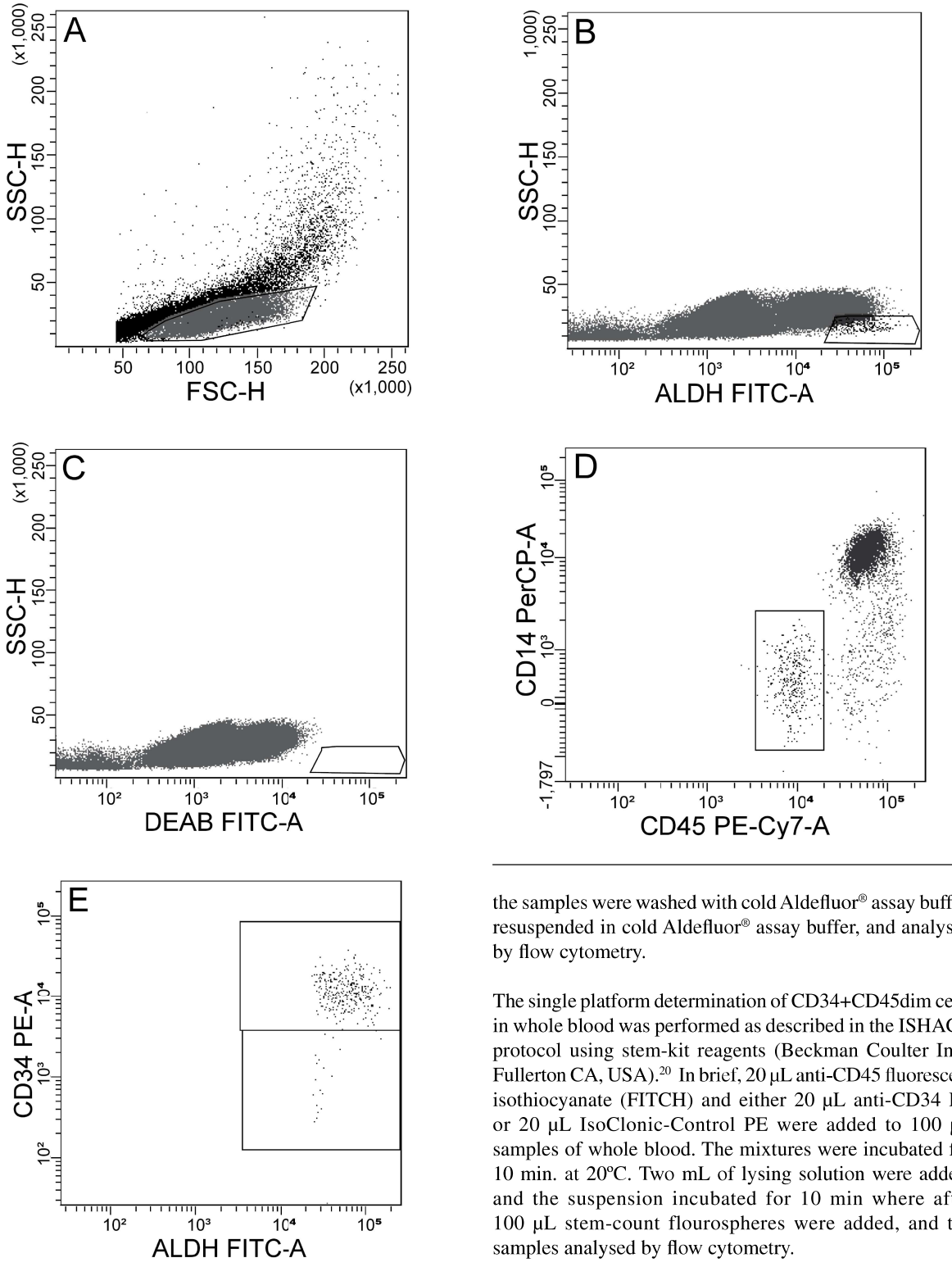
Table 2
Patient comorbidities, smoking and medications

No.	Smoking	Comorbidity	Medication
1	No	Heart disease	Salicylic acid, isoptine retard, simvastatine, venlafaxine, eltroxine
2	Yes	None	None
3	Yes	Cerebral embolism	Morphine, amlodipine, centyl with potassium chloride, plavix, paracetamol, tradolan, simvastatin
4	No	None	None
5	No	Heart disease	Digoxin, salicylic acid, centyl with potassium chloride, furosime with potassium, pantoloc
6	No	None	None
7	No	None	Dolol, paracetamol
8	Yes	Hypertension	Atenolol, corodil, halcion
9	Yes	Hypertension	Paracetamol, endofen, calepracan, codeine, amlodipine, femar, calcium
10	No	None	Paracetamol, ibuprofen

following antibodies from BD Biosciences (San Jose CA, USA): phycoerythrin (PE) labelled anti-CD34, IsoClonic-Control-PE, peridinin chlorophyll protein (PerCP) labelled anti-CD14, and PECyanin7 (PECy7) labelled anti-CD45. Staining was performed according to the manufacturer's protocol. In brief, 1.5 x 10^6 mononuclear cells were resuspended in 1.5 mL Aldefluor® assay buffer. Five µL of activated Aldefluor® reagent were added, and 0.5 mL of the suspension was transferred to each of the two control tubes containing 5 µL N,N-diethylaminobenzaldehyde (DEAB) aldehyde dehydrogenase blocking reagent. The tubes were incubated at 37ºC for 30 min and centrifuged at 300 x g for 5 min, and the cell pellets were resuspended in 55 µL cold Aldefluor® assay buffer and incubated at 4ºC for 30 min. Additionally, 20 µL anti-CD34 PE were added to the first two tubes, 20 µL IsoClonic-Control PE to the third tube, and 5 µL anti-CD45 PECy7 and 5 µL anti-CD14 PerCP were added to all three tubes before incubation. After incubation

Figure 1

Dot plots of gating strategies used in the analysis of Aldefluor® stained MNCs. MNCs were gated (Figure 1A). Next, the Aldefluor® positive cells were gated (Figure 1B). As a specific control for ALDH activity, a blocking agent (DEAB) was added (Figure 1C). The Aldefluor® positive cells were gated to separate them from the Aldefluor® CD14 positive monocytes (Figure 1D). The gated cells were analysed in Figure 1E to enumerate the CD34 positive cells and the CD34 negative cells, respectively, among the Aldefluor® positive cells



the samples were washed with cold Aldefluor® assay buffer, resuspended in cold Aldefluor® assay buffer, and analysed by flow cytometry.

The single platform determination of CD34+CD45dim cells in whole blood was performed as described in the ISHAGE protocol using stem-kit reagents (Beckman Coulter Inc., Fullerton CA, USA).[20] In brief, 20 µL anti-CD45 fluorescein isothiocyanate (FITCH) and either 20 µL anti-CD34 PE or 20 µL IsoClonic-Control PE were added to 100 µL samples of whole blood. The mixtures were incubated for 10 min. at 20ºC. Two mL of lysing solution were added, and the suspension incubated for 10 min where after 100 µL stem-count flourospheres were added, and the samples analysed by flow cytometry.

Flow cytometry

Analyses of the MNC suspensions were performed on a BD FACS Canto flow cytometer (BD Biosciences, San Jose CA, USA). The gating strategy for the enumeration of the aldeflour positive stem/progenitor cells is shown in Figure 1.

The gating strategy for CD34+CD45dim cells followed the guidelines from the ISHAGE protocol either as a single platform analysis for the whole blood method or a double platform analysis for the lymphoprep isolated MNCs. For both methods, we used a fluorescence minus one (FMO) gating strategy with isotype controls instead of unmarked cells to further take unspecific binding into consideration.

For the determination of the CD34+CD45dim cells, the gated MNCs were further analysed in a side scatter (SSC)/CD34 PE plot. The CD34+ cells were gated and analysed in a CD34 PE/CD45 PECy7 plot in which the CD45dim cells were gated and counted. The control tube with IsoClonic-Control PE instead of anti-CD34 PE was analysed in the same way to define the gates and correct for unspecific binding. The lymphocytes were gated in the SSC/forward scatter (FSC) plot and the number of lymphocytes were used as the denominator in the calculation of the relative amount of CD34+CD45dim cells.

The flow cytometry of whole blood samples was performed on a Coulter Navios flow cytometer (Beckman Coulter Inc., Fullerton CA, USA). Leucocytes were gated in an FSC/SSC plot, and the gated cells were analyzed in a CD34/SSC plot. The gated CD34+ cells were analyzed in a CD34/CD45 plot, and the CD34+CD45dim cells were gated and counted. The control sample with IsoClonic-Control PE was analysed in the same way to define the gates and correct for unspecific binding. The CD34+CD45dim counts were converted to absolute concentrations based on the equivalent counts of flourosphere beads according to the manufacturer's instructions.

PLATELET FUNCTION

Platelet activation

Platelet activation was investigated by measuring the surface marker CD62P (from α granules) and binding of PAC1 (antibody that binds specifically to the activation induced conformational epitope on GPIIb-IIIa). EDTA stabilized whole blood samples were incubated with the following conjugated antibodies purchased from BD Biosciences (San Jose, CA): PAC1 FITCH, anti-CD62P PE, anti-CD41 PE-Texas red (ECD) and anti-CD45 PE-Cyanin 5.1 (PC5). Relevant isotype control antibodies were used as controls. The samples were analysed by flow cytometry (Coulter Navios, Beckman Coulter Inc.). Platelets were identified in logarithmic/SS plots with 10000 CD41+ counts in the platelet gate. The results are presented as proportion of stained cells as defined by the appropriate isotype control.

Thromboelastography (TEG)

Clot formation was assessed in citrated whole blood using a TEG 5000 Haemostasis Analyser System (Haemonetics Corp, Braintree MA, USA) according to the manufacturer's recommendations. The variables reported were reaction time (R – reflecting rate of initial fibrin formation), alpha angle (α – reflecting clot formation kinetics) and maximum amplitude (MA – reflecting maximum clot strength).[21]

Multiplate assay

Whole blood platelet aggregometry was assessed using the Multiplate® (Dynabyte Medical, Munich, Germany) device measuring increased impedance (expressed as the area under the curve (AUC) of increasing impedance over time) in whole blood as a consequence of aggregation of stimulated platelets on the electrodes.[22] Platelets were stimulated according to the manufacturer's instructions with adenosine-diphosphate (ADP, final concentration 6.5 $\mu mol \cdot L^{-1}$), arachidonic acid (ASPI, final concentration 0.5 $mmol \cdot L^{-1}$) and thrombin receptor-activating peptide 6 (TRAP, final concentration 32 $\mu mol \cdot L^{-1}$).

STATISTICAL ANALYSIS

The difference between pre- and post-HBO treatment values of CD34+CD45 dim cells, of Aldefluor® positive cells, and of the platelet activation and function tests were tested for significance by the Wilcoxon matched-pairs signed-ranks test. $P > 0.05$ was considered not significant (n.s.). The putative cumulative effect of the first 15 HBO exposures on the CD34+CD45dim counts was analysed with a linear mixed effects model using Statistical Analysis System (SAS/STAT 9.2) software.

Results

Six of 10 enrolled patients (1, 2, 4, 6, 7 and 10) completed the full treatment course (29–30) within a period of 42–49 days. For patients 1, 2 and 10, the duration beyond six weeks (42 days) was due to illness or cancellation of treatments due to acute situations. Four patients (3, 5, 8 and 9) dropped out at some point after the fifteenth exposure or did not comply with the study treatment period (a duration of more than 50 days in total was considered as non-compliance, which was the case for patients 5 and 9). The whole blood counting of CD34+CD45dim cells around the first HBO exposure of patient 9 failed, and thus patient 9 is excluded from Figure 2. Reasons for non-compliance were lack of physical and mental energy.

MEASUREMENT OF CD34+CD45dim CELLS

The results given are the mean of duplicate measurements. Results from the single platform whole blood analyses of absolute numbers of CD34+CD45dim cells are shown in Figure 2. We found a tendency for a rise in CD34+CD45dim

Figure 2

CD34+CD45dim cells per mL blood. Results from absolute counting in whole blood samples. Significance tests are the results from Wilcoxon matched-pairs signed-ranks tests for difference between results on adjoining vertical lines (n.s. = not significant)

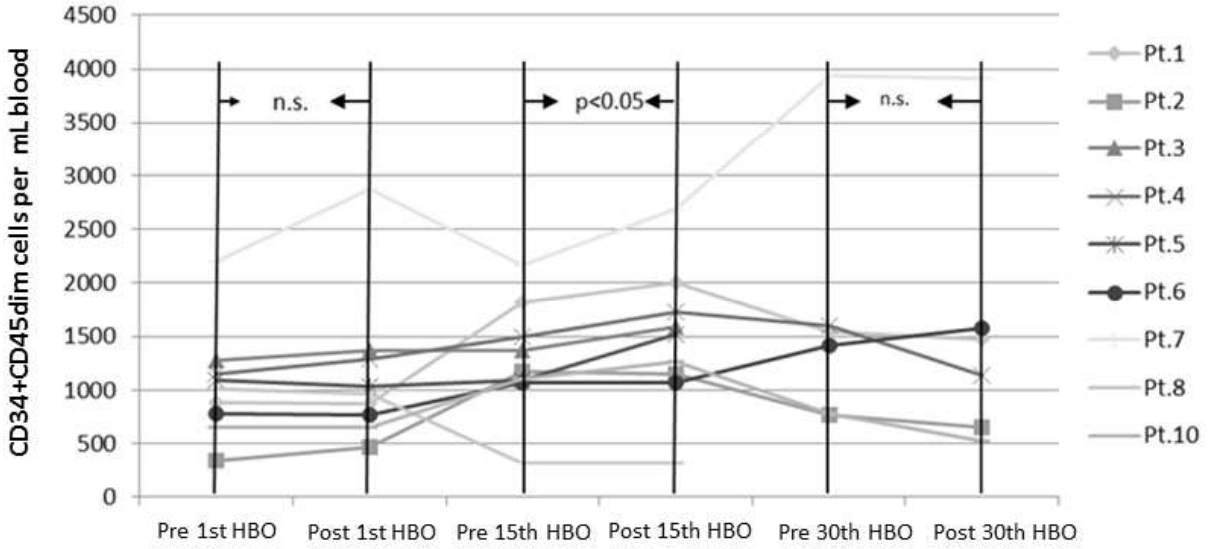
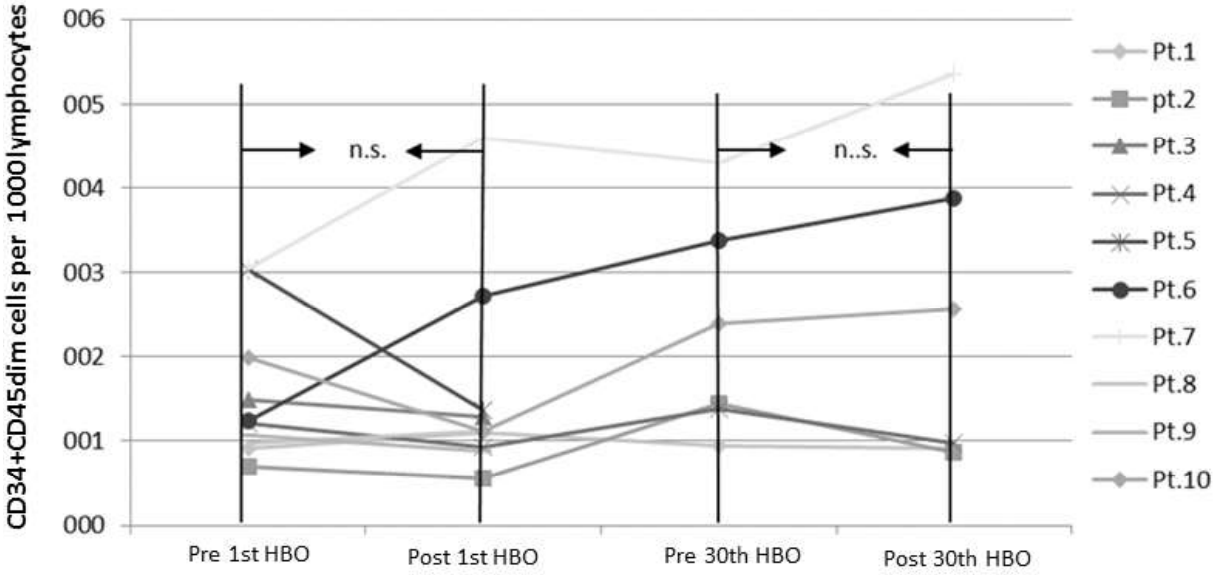


Figure 3

CD34+CD45dim cell fraction of lymphocytes. Results from analysis of the mononuclear cell suspensions. Significance tests are the results from Wilcoxon matched-pairs signed-ranks tests for difference between results on adjoining vertical lines (n.s. = not significant)



counts from before to immediately after a given HBO exposure. However, except for around the fifteenth exposure, the changes did not reach statistical significance, and after the thirtieth exposure there was a small reduction in CD34+CD45dim counts. In the six patients who completed the full treatment course, there was a rise in CD34+CD45dim counts from before the first to after the thirtieth exposure but it was weak and did not reach statistical significance. The dropping-out of four of 10 patients precluded a valid statistical analysis of the results after the thirtieth HBO exposure since a drop-out bias cannot be excluded. However, all patients completed 15 HBO exposures, and we found a significant rise in CD34+CD45dim counts after the fifteenth versus before the first exposure using a linear mixed effect model (Table 3). The upper 97.5% limit of the rise in CD34+CD45dim counts was 931 cells·ml^{-1}, i.e., below a doubling of the counts recorded before the first HBO exposure, the mean of which was 1101 CD34+CD45dim

Table 3

Analysis of the CD34+CD45dim number of cells per mL blood around the first and the fifteenth HBO treatment with a linear mixed effects model with fixed effects for run (1 or 15) and treatment (pre or post) and their interaction. The random effects follow a variance component structure with random effects for patients and patient and run combinations. Missing data are assumed to be missing at random. The mean number of CD34+CD45dim cells per mL blood before the first HBO treatment was 1101. Comparisons, single-step adjusted for multiple comparisons. 2.5% and 97.5%, lower and upper confidence interval limits

Comparison	Estimate	2.5%	97.5%	*P*-value
Post 15 – pre 1	498.39	65.08	931.71	0.018
Post 1 – pre 1	117.27	-22.43	256.96	0.128
Post 15 – pre 15	201.11	47.42	354.80	0.005

Table 4

Platelet function by multiplate analyzer and thromboelastograph. ADP = adenosine triphosphate; ASPI = arachidonic acid; AUC = area under curve; MA = maximal amplitude; R = reaction time(s); TRAP = thrombin receptor activating peptide 6

Analysis	% change after versus before 1st HBO treatment	
	Mean (SD)	Wilcoxon
Multiplate Analyzer		
ASPI (AUC)	20 (45)	n.s.
ADP (AUC)	-4.4 (23)	n.s.
TRAP (AUC)	6.5 (24)	n.s.
Thromboelastograph		
R (s)	0.7 (21)	n.s.
Alpha angle (degree)	0.05 (5.8)	n.s.
MA (mm)	2.2 (5.7)	n.s.

cells·mL^{-1}. However, the lower 2.5% limit of the rise in CD34+CD45dim counts was 65, which is compatible with the notion that repeated HBO exposures might induce a modest rise in the CD34+CD45dim counts.

The changes in the CD34+CD45dim fraction of lymphocytes from before a given HBO exposure to immediately after varies between a modest rise in some patients and a modest fall in others (Figure 3). However, neither the changes around the first HBO exposure nor the changes around the thirtieth exposure reach statistical significance, thus confirming the results from the analysis of whole blood.

The mean (SD) change in CD34+CD45dim cells measured immediately after versus before a given HBO exposure was 4.2% (17%) (n = 23) in the absolute CD34+CD45dim measurements and 0.2% (42%) (n = 16) in the relative CD34+CD45dim measurements. Neither of these changes were significantly different from 0%.

MEASUREMENT OF ALDEFLUOR POSITIVE CELLS

The changes in Aldefluor® positive cells expressed as fraction of lymphocytes are shown in Figure 4. We found that the second of the duplicate measurements of Aldefluor® positive cells was significantly lower, 51% (21%), than the first measurement, presumably due to leakage from the cells of some of the ALDH converted intracellular Aldefluor® during the time lapse of 30–45 minutes between sample analysis. Thus, the results given are from only the first of the duplicate measurements. Only insignificant changes are imposed by the first HBO exposure (10 patients) and the thirtieth HBO exposure (six patients). The CD34-negative subpopulations of Aldefluor® positive cells, including possible MSCs, are given as white columns in Figure 4. Only a minute fraction of Aldefluor® positive cells was CD34 negative, and it was not significantly influenced by the HBO treatment.

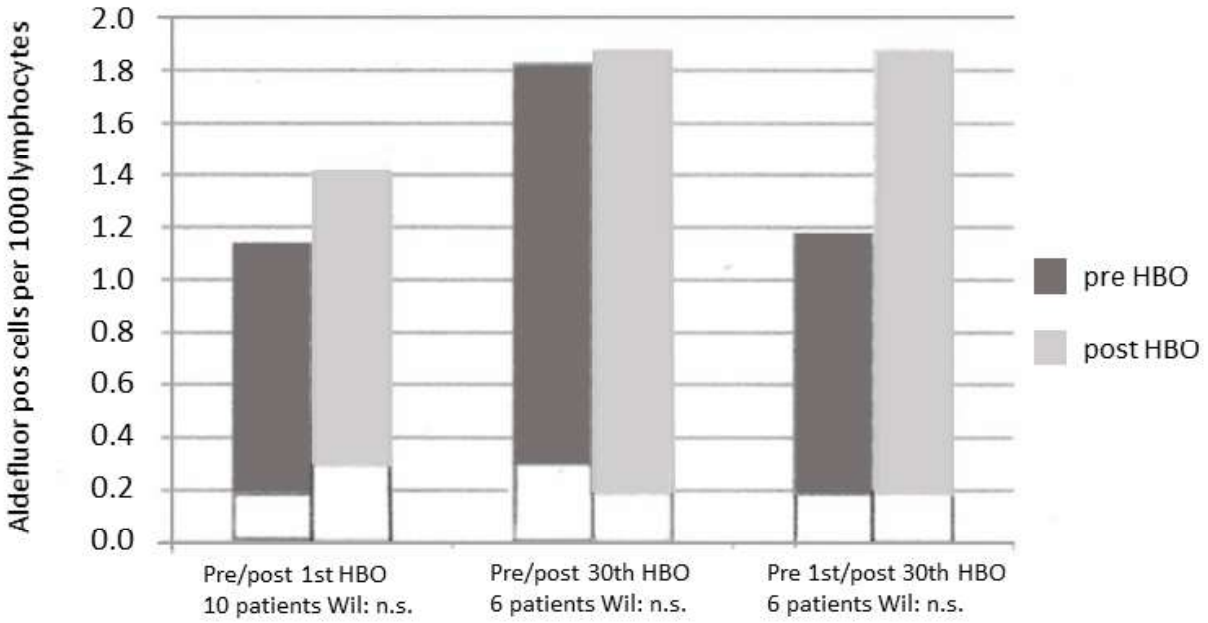
Platelet measurements

Results from measurements of platelet function before and after the first HBO exposure are shown in Table 4. The multiplate analyzer results indicate that no statistically significant changes were seen in aggregation efficiency after various activators as measured by the AUC. Additionally, no statistically significant changes were seen after versus before the first HBO exposure in the clot formation as measured by the thromboelastograph.

In the flow cytometric measurement of activation markers shown in Figure 5, we could not detect a statistically significant change in the fraction of platelets expressing the activation markers post-HBO versus pre-HBO taking all ten patients into consideration. However, excluding patients 4, 5, and 9 who all had pretreatment values below 2% CD62 positive platelets and below 5% PAC positive platelets, the remaining seven patients had a statistically significant fall in the fraction of activated platelets ($P < 0.02$ for both activation markers).

Figure 4

Aldefluor® positive cell fraction of lymphocytes. Results from analysis of the mononuclear cell suspensions. Wil: n.s. stands for: Wilcoxon matched-pairs signed-ranks tests for difference between results in adjoining columns (n.s. = not significant). The white areas at the bottom of each column indicates the amounts of the Aldefluor® positive cells which were CD34 negative



Discussion

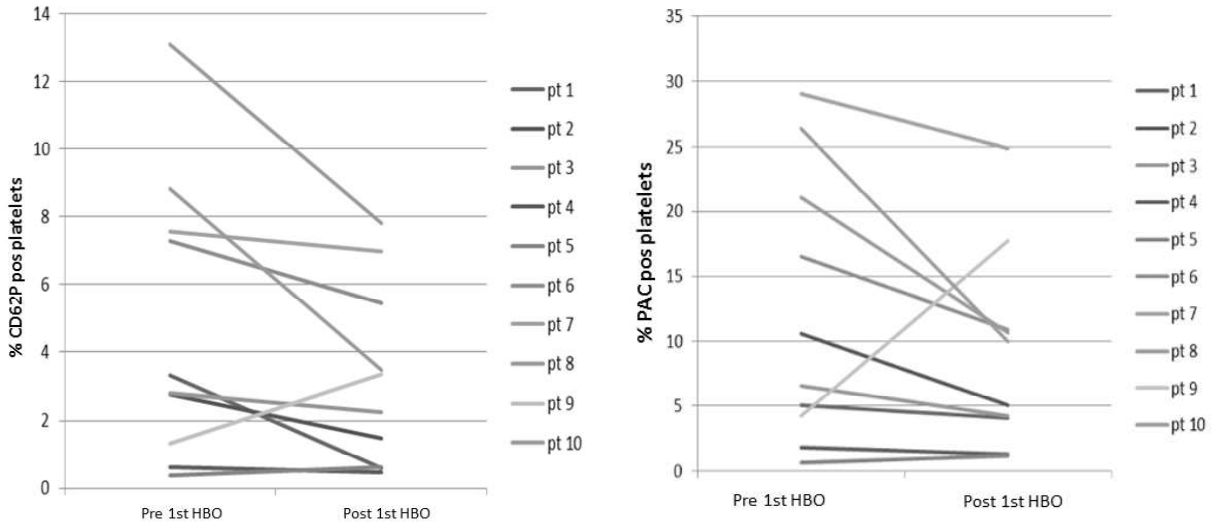
The present study demonstrated a weak and insignificant tendency toward mobilization of CD34+CD45dim cells after a single exposure to 2.4 ATA HBO in both absolute and relative values. Because four patients did not complete the scheduled 30 HBO exposures, a putative additive effect of the preceding HBO exposures could only be analyzed in a statistically valid way by comparing the absolute CD34+ counts before the first HBO exposure to the CD34+ counts after the fifteenth HBO exposure. In this case, it was less than a doubling but still more than zero.

The drop-out of four patients in the present study may well have impacted the results due to decreased statistical power, which may have contributed to the contrast between our findings and two of the studies by Thom's research group.[4,10] In 2006 and 2014, they reported, in larger patient populations qualitatively similar to the present one, a more than doubling of the CD34+CD45dim population after only one HBO exposure.[4,10] Also, they found increases of approximately 8- and 20- fold after 20 HBO exposures at 2.0 and 2.5 ATA, respectively. In 2011, Thom et al.[11] reported a more than doubling after one HBO exposure in patients with diabetic ulcers, but, in accordance with the present study, they did not find a cumulative effect of repeated exposures (up to 20 exposures) as in the other two studies. This difference from the two previous studies was interpreted by the authors as caused by different diagnoses. For completeness, another study investigated HBO treatment in chronic wound patients and reported increases in values of circulating CD34 cells relative to blood cells of the same order of magnitude as the findings by Thom et al. No absolute values were given.[23]

In contrast, Shandley et al., reported only a non-significant trend towards increasing stem cell percentage with increasing number of HBO treatments after traumatic brain injury and correlated CD34+CD133+nestin+cells with different outcome measures of cognitive performance.[24] The authors found that improvement in clinical results correlated with stem cell mobilization in the HBO treated group, but not in the sham treated group. However, although, the values of circulating SPCs are difficult to interpret and no definite values are given, overall, they only found minor and non-significant trends towards increasing stem cell percentage with increasing number of treatments. Also, it appears that for both HBO and sham treated patients, there was an approximately equal distribution between increase/no change/decrease in stem cell mobilization. Thus, their conclusions regarding circulating SPCs are in line with our findings.

The contradictory findings between the studies are hard to explain. It could be argued that different diseases could possibly affect the results, an assumption supported by the studies by Thom et al. Nevertheless, even in similar patient populations, the reported data are very different. Although undocumented, there are several unknown factors that could possibly affect the results, e.g., the kinetic and circulation time of cells potentially recruited into the development of

Figure 5
Fraction of activated platelets. Flow cytometric analysis of the fraction of platelets expressing the activation markers: CD62P and PAC-1 positivity, pre and post first HBO treatment



new tissue and the possible influence of the frequency of change in oxygen as well as the overall dose and delivery regimen. Also, one could speculate that the use of air breaks during HBO treatment sessions, creating an oscillation in tissue oxygen partial pressure, could have an impact on stem cell responses as normobaric oxygen oscillation has been shown to affect hypoxia-inducible factor 1a (HIF-1a) and erythropoietin (EPO) expression levels.[25] We did not use frequent air breaks in our setting. In the related studies, use of air breaks is not mentioned.[4,10,11]

In addition, differences/inconsistencies in the reporting make the results difficult to interpret and compare, e.g., the lack of absolute values,[23,24] a factor 8 to 10 difference in CD34+ counts at baseline ranging from 0.2% of lymphocytes to 0.2% of whole blood cells (WBC)[4,10,11] and statements regarding surface marker expression on EPCs versus HSCs that contradicts the guidelines from ISHAGE.[4,20]

In the present study, we used two different methods – a MNC separation technique and a whole blood method – to determine the CD34+CD45dim population and found results that were consistent between the methods. In addition, we detected cells with high ALDH activity as a marker for stem cells of any kind. We demonstrated a trend towards an approximately 50% increase in ALDH positive cells from before the first to after the thirtieth HBO exposure, although the increase was not statistically significant. Again, the drop-outs reduced the statistical power, but still our observations are very far from the 8- or 20-fold increase in circulating SPCs described in some previous studies.[4,10] Four of the ten patients in our study were smokers, which may possibly have contributed to the overall results. Cigarette smoke extract has been demonstrated to inhibit MSC migration and differentiation in vitro,[26] and cigarette smokers have lower EPC numbers in circulating blood and bone marrow post-acute myocardial infarction.[27]

With respect to the putative EPCs, it should be noted that the biological significance of circulating bone marrow derived EPCs recently has been questioned. Thus, genetic fate mapping has shown that the endothelial stem/progenitor cells involved in adult angiogenesis are not bone marrow derived but derive from local tissue resident cells.[28] Furthermore, Fang et al.[29] have provided evidence for adult vascular endothelial stem cells that reside locally in the blood vessel wall endothelium.

There have been diverging results regarding whether HBO may induce a hypercoagulation state and/or platelet activation.[15–18] In the present study, we used a whole blood viscoelastic hemostatic test, the TEG, a whole blood WB aggregometry analyzer, the Multiplate, and flow cytometric detection of platelet activation markers. In general, we found no alterations in any of these tests comparing post-HBO values to pre-HBO values. Interestingly, seven of the ten patients had at baseline more than 2% CD62P positive platelets and more than 5% PAC-1 positive platelets, reflecting increased platelet activation before the first HBO exposure. In these patients, we found a significant decrease in activated platelets after the first HBO exposure. The result is only indicative of a possible action of HBO and should be confirmed in a larger study. However, if HBO treatment indeed decreases a heightened platelet activation state, the treatment would hamper the migration of activated platelets to areas of inflammation. No concomitant alterations were detected in the functional tests, possibly because of lack of sensitivity at this level.

Conclusions

We found a minor statistically significant mobilizing effect on the bone marrow derived stem/progenitor cell content in peripheral blood after 15 HBO treatments (n = 10 patients), but no effect after 30 treatments (n = 6 patients). However, because of the low number of patients we cannot confidentially prove or disprove the null hypothesis. The possibility that HBO treatment reduces the number of activated platelets could not be demonstrated nor excluded.

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