The importance of hypoxia and extra physiologic oxygen shock/stress for collection and processing of stem and progenitor cells to understand true physiology/pathology of these cells \textit{ex vivo}

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**Purpose of review**
Hematopoietic stem (HSCs) and progenitor (HPCs) cells reside in a hypoxic (lowered oxygen tension) environment, \textit{in vivo}. We review literature on growth of HSCs and HPCs under hypoxic and normoxic (ambient air) conditions with a focus on our recent work demonstrating the detrimental effects of collecting and processing cells in ambient air through a phenomenon termed extra physiologic oxygen shock/stress (EPHOSS), and we describe means to counteract EPHOSS for enhanced collection of HSCs.

**Recent findings**
Collection and processing of bone marrow and cord blood cells in ambient air cause rapid differentiation and loss of HSCs, with increases in HPCs. This apparently irreversible EPHOSS phenomenon results from increased mitochondrial reactive oxygen species, mediated by a p53-cyclophilin D–mitochondrial permeability transition pore axis, and involves hypoxia inducing factor-1α and micro-RNA 210. EPHOSS can be mitigated by collecting and processing cells in lowered (3%) oxygen, or in ambient air in the presence of, cyclosporine A which effects the mitochondrial permeability transition pore, resulting in increased HSC collections.

**Summary**
Our recent findings may be advantageous for HSC collection for hematopoietic cell transplantation, and likely for enhanced collection of other stem cell types. EPHOSS should be considered when \textit{ex-vivo} cell analysis is utilized for personalized medicine, as metabolism of cells and their response to targeted drug treatment \textit{ex vivo} may not mimic what occurs \textit{in vivo}.

**Keywords**
extra physiologic oxygen shock/stress and its mitigation, hematopoietic stem and progenitor cells, microenvironment, oxygen tension
Hematopoiesis

**KEY POINTS**

- HSCs and HPCs reside in vivo in a microenvironment that is quite hypoxic compared with that of ambient atmospheric O₂.
- Collection and processing of mouse bone marrow and human cord blood in ambient air underestimate numbers of HSCs in these tissues due to EPHOSS that is triggered by higher O₂ than that found in vivo. This results in increased production of mitochondrial ROS and differentiation of HSCs.
- EPHOSS appears to be irreversible and mediated by a p53-CypD–MPTP axis, and is associated with expression of hif-1α and miR210.
- Mitigation of EPHOSS by collection and processing of bone marrow and cord blood in hypoxia, or alternatively in air in the continuous presence of CSA, results in higher numbers of HSCs.

and cell cycling of colony-forming unit (CFU)-granulocyte macrophage, CFU-granulocyte, CFU-macrophage, burst-forming unit (BFU)-erythroid, CFU/BFU-megakaryocytic, and multipotent (CFU-granulocyte erythroid, macrophage, megakaryocyte, CFU-Mix) HPCs when in vitro culture conditions are hypoxic. Expansion of HPCs and HSCs ex vivo is superior under hypoxic culture conditions [15,17].

Studies have evaluated the distribution of HSCs and HPCs in relationship to bone marrow micro-environmental cells in the context of regional O₂ levels. HSCs and cells within bone marrow that support HSCs are mainly present in a niche predominately located at a lower region of the O₂ gradient, suggesting that regional hypoxia plays an important role in regulating HSC function [5]. More recent studies have refined concepts of HSC localization. One study defined HSC phenotype within endosteal bone marrow regions as being superior for homing and proliferative capacity, compared with these same phenotyped cells isolated from the central bone marrow [18]. Another group performed in-vivo measurements of local O₂ tension in live mice [7**] using two-photon phosphorescence lifetime microscopy to determine that absolute local O₂ tension of the bone marrow was low (<32 mmHg) even though there was a very high vascular density. Although the bone marrow as a whole was hypoxic, they found heterogeneity in local O₂ levels with the lowest (about 9.9 mmHg, or 1.3% O₂) present in deeper perisinusoidal regions. Under conditions of post-chemotherapy stress, HSCs and HPCs did not seek out specific niches defined by low O₂ for their preferential homing. Another group used five-color imaging cytometric analysis to quantitate the distribution of HSCs and HPCs in femoral bone marrow cavities [6]. HSCs and HPCs localized preferentially in endostal zones, in which they interacted closely with sinusoidal and non-sinusoidal bone marrow microvessels. HSCs/HPCs exhibited a hypoxic metabolic profile defined by strong retention of pimoni-dazole and expression of hypoxia inducing factor-1α (hif-1α), regardless of location in the bone marrow, position next to vascular structures, or cell cycle state. Thus, the hypoxic phenotype of HSCs and HPCs in bone marrow was cell, rather than location, specific. Endosteal bone marrow areas did not contain the most hypoxic HSCs/HPCs, and hif-1α stabilization in these cells occurred independent of differences in O₂ levels at different anatomical sites.

**EXTRA PHYSIOLOGIC OXYGEN SHOCK/STRESS**

Although the biology of HSCs/HPCs and other stem cells (embryonic, mesenchymal, and neural) are now considered in the context of anatomical site positioning in vivo, and in context of O₂ tension for growth differences ex vivo [8,19], no attempts have been made to assess initial effects of even brief exposure of HSCs and HPCs to ambient atmospheric O₂ regardless of whether or not the cells collected in ambient air are subsequently processed, cultured, or injected into animals under normoxia or hypoxia. Our most recent studies [20**] now demonstrate that even very brief exposure to ambient air has a rapid and apparently irreversible effect that changes the metabolism of HSCs and HPCs. Through a phenomenon that we termed extra physiologic oxygen shock/stress (EPHOSS), this results in rapid loss of HSC numbers with concomitant increases in HPCs, because of rapid differentiation of HSCs. Mechanisms of EPHOSS encompass ambient air-induced production of mitochondrial reactive oxygen species (ROS), and induction of the mitochondrial permeability transition pore (MPTP) opening. This occurs with bone marrow and also human cord blood cells, which is consistent with reports that human cord blood cells are also in a hypoxic environment [21]. EPHOSS is mediated by interactions with the MPTP and cyclophilin D (CypD) and p53, with links to expression of hif-1α, and the hypoxamir, micro-RNA 210 (miR210). This information is important for hematopoietic cell transplantation (HCT), especially for cord blood HCT in which numbers of cells from single collections are low. Although efforts have focused on enhancing the current clinical efficacy of these cells for HCT via ex-vivo expansion of these cells, or by increasing their homing capabilities [22,23], to compensate...
low collection numbers, being able to collect more HSCs in a cord blood collection could greatly enhance the efficacy of cord blood for HCT. In fact, EPHOSS, and means to prevent its action, will likely extend to many other stem cell types, including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), adipose stem cells (HSCs), and other tissue specific stem cells that normally reside in a hypoxic environment in vivo.

ROS can be toxic, but also has differentiation inducing activity [24\*]. We reasoned that collecting mouse bone marrow cells and doing all processing and eventual culture of the cells or their injection into mice under low O\(_2\) tension might mitigate production of mitochondrial ROS, and subsequent ROS-induced differentiation of the mouse bone marrow HSCs [20\*]. For this to be successful, it was necessary for all procedures to be done in a hypoxic chamber in which everything used (media, plastic ware, glassware, syringes, etc.) was preequilibrated to 3% O\(_2\) in the hypoxic chamber for 18 h prior to collection, processing, and eventual culture or injection into mice under 3% O\(_2\). This resulted in approximately three-fold to five-fold enhanced collection of mouse bone marrow long-term repopulating-HSCs, rigorously determined by phenotype and functional engraftment of competitive repopulating units as defined by donor cell chimerism and limiting dilution analysis, with concomitant decreases in HPCs, defined by phenotype for short-term repopulating-HSCs and multipotential progenitor cells, and by function using colony assays for CFU-granulocyte macrophage, BFU-erythroid, and CFU-granulocyte erythroid, macrophage, megakaryocyte.

Collection of cells in 3% O\(_2\) and then placing them in ambient air for as short as 20–30 min (the shortest time in which we were able to process the cells) resulted in greatly reduced numbers of HSCs and increased numbers of HPCs. Additionally, collection and processing of cells in air, or collecting of cells at 3% O\(_2\) and placement in ambient air greatly increased production of mitochondrial ROS, mitochondrial mass/activity, and high mitochondrial membrane potential. EPHOSS did not link to apoptosis, nor did it influence the homing efficiency of the collected cells. Collection and processing of human cord blood CD34\(^+\) cells in 3% O\(_2\) also resulted in about a three-fold increase in rigorously defined human HSCs [25], demonstrating that effects of EPHOSS were not limited to bone marrow [20\*].

**MECHANISMS OF EXTRA PHYSIOLOGIC OXYGEN SHOCK/STRESS**

We evaluated mechanisms of EPHOSS for obtaining insight into its biology, and also for potential alternative means of collecting HSCs in order to mimic the enhancing effects of HSC collection at low O\(_2\) tension [20\*]. Collection of bone marrow or cord blood cells at low O\(_2\) would present a logistical problem that even if solved would make collection of cells cumbersome and expensive. We focused on the MPTP as a potential key to EPHOSS [20\*]. Although oxidative stress favors induction of the MPTP opening, which can result in the swelling of mitochondria, and uncoupling of OXPHOS that leads to apoptosis and necrosis [26,27], this MPTP opening can be transient and function in a regulatory capacity conducive to modulating differentiation of stem cells. A key regulatory component of the MPTP is CypD, which regulates induction of the MPTP [28,29]. Interestingly, cyclosporine A (CSA), a small molecule inhibitor of CypD that binds CypD and antagonizes induction of the MPTP [30,31], is Federal Drug Administration approved and is used as an immuno-suppressant to treat graft versus host disease for HCT, as well as a treatment possibility for heart attack and stroke [32,33]. We reasoned that CSA might be useful to protect against effects of MPTP induction, and if this was successful it might be rapidly considered for the collection of HSCs in ambient air by mimicking effects of low O\(_2\) tension. We found that collection and processing of mouse bone marrow or human cord blood in ambient air but in the immediate and continued presence of CSA resulted, respectively, in greatly enhanced numbers of phenotypically identified HSCs and functional competitive repopulating units for mouse bone marrow, and severe combined immunodeficiency-repopulating cells for human cord blood [20\*]. To maintain HSC numbers in cord blood through the cryopreservation and thaw procedures necessary for cord blood banking, it is likely that CSA may have to be present throughout the freeze/thaw procedures.

To implicate the MPTP in EPHOSS further, we assessed whether or not CypD deletion (+/−), which is known to prevent induction of the MPTP [34–36], might protect against effects of EPHOSS for enhanced collection of HSCs from mouse bone marrow. CypD −/− mouse bone marrow cells collected and processed in air were greatly increased in phenotypically defined and functional HSCs, with decreased numbers of HPC compared with CypD +/+ mouse bone marrow. CypD −/− bone marrow long-term repopulating-HSC was also significantly reduced in production of mitochondrial ROS. Evaluating mouse CypD −/− spleen cells by the Seahorse XF96 flux analyzer demonstrated that basal respiration and maximal respiratory capacity was higher in CypD −/− cells than in wild-type control cells [20\*].

We were also able to link p53 −/− bone marrow cells to a p53−CypD−MPTP axis in mechanisms of
EPHOS [20**]. Using *hif-1α* and *miR210*−/− mouse bone marrow cells, we also linked *hif-1α* and *miR210* to EPHOS [20**], although exact mechanisms have not yet been worked out. *CypD*−/− and *p53*−/− had EPHOS-protective effects, wherein *hif-1α*−/− and *miR210*−/− abrogated the protective effect seen under hypoxic harvesting and processing of the cells. Our studies highlight how interpretation of experimental results of mouse gene deletion models can be influenced once EPHOS is considered.

**BROAD IMPLICATIONS FOR ROLE OF EXTRA PHYSIOLOGIC OXYGEN SHOCK/STRESS IN INTERPRETATION OF STUDIES OF HEMATOPOIETIC STEM CELLS/HEMATOPOIETIC PROGENITOR CELLS AND OTHER STEM/PROGENITOR CELL TYPES**

Our studies on EPHOS [20**] clearly link this phenomenon to HSCs, HPCs, and regulation of hematopoiesis. However, we believe that this phenomenon has much broader implications, not only for understanding the potential true in-vivo numbers, characterization, and function of HSCs and HPCs, but also in the context of development and pathologic cell types. Many types of adult stem cells exist naturally in niches *in vivo* that are hypoxic [37], and ESCs, which are found in the inner mass of blastocytes, and cancer stem cells (CSCs) reside in hypoxic environments [38–40]. ROS is important in the growth, differentiation, and the regulation of these cells [24,41–43]. Ex-vivo growth in lowered O₂ tension favors the growth of ESCs, induced pluripotent stem cells, CSCs, as well as MSCs, ASCs, and other cells [24,44–47]. Although much has been written about the metabolism of HSCs, HPCs, ESCs, CSCs, and MSCs among a plethora of other stem/progenitor cell types [48,49–53], critical consideration should now be given as to how accurate these measurements and analyses are with regards to the metabolism of these cells and their function *in vivo* in hypoxic environments. Thus, many studies on metabolism of stem/progenitor cells may have to be reevaluated in context of EPHOS. This is especially of relevance for future efforts of personalized medicine, as such treatments would be based on gene expression patterns and response of a person’s tissue to ex-vivo treatment. However, metabolic profiling for the development of specific therapeutic strategies meant to target, for example, CSCs [54–56] may not accurately represent the metabolism of these cells as they exist in their microenvironment *in vivo*, as these cells are harvested and studied in atmospheric oxygen, and have already been subjected to consequences of EPHOS.

Another area to consider for effects of EPHOS would be aging and senescence and its effects on the

![Figure 1](image-url)
metabolism, and response of stem cells from aged animals or humans to cytokines/growth modulating factors. Aging has detrimental effects on HSCs and many other tissue-specific stem cells [57–65]. As ROS has been linked as a driver in the aging process, it is possible that stem cells from aged animals and humans may be especially susceptible to EPHOSS-linked production of mitochondrial ROS after collection and processing of these cells in ambient air. The therapeutic potential of even aged HSCs may be enhanced if EPHOSS is mitigated during their collection/harvest.

Many factors influence the regulation of stem/progenitor cells in vivo. For example, the enzyme, Dipeptidylpeptidase (DPP4) which can truncate and change the functional activity of a large number of cytokines/growth factors, and other growth modulating proteins [66–69]. DPP4 is found within cells and in the serum, and is also present on cell surfaces of HSCs, HPCs, mature hematopoietic cells, and other cells such as CD26. How DPP4 works in vivo and in context of EPHOSS remains to be determined.

CONCLUSION

EPHOSS is a new, interesting, and likely important phenomenon which will have relevance to understanding the true physiology and pathology of stem and progenitor cells and how they will best be assessed for future therapeutic modalities [20**]. Information on HSCs, HPCs, and other stem and progenitor cells and their interactions with micro-environmental niche cells, which have reached extremely high levels of sophistication [70–73], may now also have to be reconsidered for reevaluation in the context of present and future knowledge of the effects of EPHOSS on cellular processes. EPHOSS may also influence metabolism and differentiation of lymphocytes, monocytes and granulocytes, and other mature tissue cells. Figure 1 diagrams the potential impact of EPHOSS, and implications of EPHOSS for different stem, progenitor, and more mature cell types, whether normal or from patients or animals with malignant and nonmalignant disorders, and should be considered as an important and worthy pursuit. More mechanistic insight into EPHOSS is warranted.

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Conflicts of interest

H.E.B. is a member of the Medical Scientific Advisory Board of Corduse, a public cord blood banking company, and is a Founder of the Corduse family cord blood bank. The remaining authors have no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

8. An outstanding in depth look at the oxygen status of HSC niches.
Hematopoiesis


This article has identified a new, and previously unrecognized detrimental effect of short exposure of tissue sources of HSCs to brief exposure to ambient air, as well as mechanisms involved and means to mitigate the effect for enhanced collections of HSCs, information of potential clinical relevance.


An excellent review of the role of ROS in stem cell function.


26. Halestrap AP, Davidson AM. Inhibition of Ca(2+)-induced large-amplitude swelling of liver and heart mitochondria by cyclopiazon is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 1990; 268:153–160.


