Isolation and Culture of Human Bone Marrow MSC Under Unbroken Physioxia for Better Cell Growth and Translatability of Studies

Alicia D. Henn PhD MBA, Shannon L. Darou, BS, Collins E. Gbegbeawu, MS, Syed A. Mustafa, MBA
BioSpherix, LLC. Parish, NY.

BioSpherix
part of the Breeze Group

Abstract # 3274

Background

- Human bone marrow mesenchymal stromal/stem cells (MSC) are used for *in vitro* toxicologic assays, both in traditional 2D and in 3D organoid cultures for microphysiologic systems.
- Publications by others showing that supraphysioxic room air adds artifact to *in vitro* assays have led to calls to protect cells from room air.[1,2]
- We previously showed that commercially available MSC cultures proliferate faster and grow for more passages under physioxia (5% O2) than under traditional conditions (18% O2). We also showed that HIF-1a, a key signaling molecule upstream of cell growth pathways, is modulated by room air exposure in MSC.[3]
- Human tumor cells exhibit different drug-screening results when isolated under physiologically relevant oxygen (physioxia).[4,5]

Objective and Hypothesis

- Here, we extend earlier findings by isolating the cells from cadaver bones and expanding them under constant physioxia, so that the cells are never exposed to room air oxygen.
- The null hypothesis for this study is that there will be no difference in doubling times between cells exposed to, and those protected from, room air.

Methods

- Except for commercially sourced MSC cell lines, all cell isolation and culture operations were performed under closed and controlled conditions in Xvivo Systems (BioSpherix). Centrifuges, microscopes, and other laboratory equipment were housed inside the closed modules.
- Commercial human bone marrow MSC (Lonza) were cultured at constant 5% or 18% O2 (room air incubator O2 control) plus 5% CO2 in T-flasks. Media were pre-equilibrated to the proper oxygen level. Commercially available MSC specialty media were tested for cell growth kinetics under constant physioxia.
- Cell counts were obtained with the Denovix CellDrop or manual trypan blue count.
- Cells were isolated from human cadaver pelvic bones, sourced from a licensed organ procurement organization, inside the Xvivo System for unbroken control of the cell environment. The primary bone marrow cell isolate was subjected to discontinuous centrifugal gradient separation and mononuclear cells were selected for adherence to plastic.
- To confirm MSC identity, flow cytometric analysis was done with a Cytek Aurora and the following anti-human antibody panel (Dead cell-Zombie Aqua, CD38-PACBLUE, HLA-DR-BV785, CD45-FITC, CD14-PerCP-Cy5.5, CD73-PE-Cy7, CD105-APC, CD90-APC-Cy7(Biolegend), CD11b-BUV395 (BD), CD34-PE (Thermo)).
- The cells were cultured for 2-4 weeks in specialized media (Thermo) to differentiate them into adipocytes, chondrocytes, and osteocytes. The cultures were stained with Oil Red O, Alcian Blue, and Alizarin Red S, respectively, and assessed visually (qualitatively) for cell staining.

Conflict of Interest Disclosure All authors are employees of BioSpherix, LLC.

Experimental Set Up

Figure 1: Full-Time Environmental Control
The BioSpherix Xvivo System® is a HEPA-filtered, closed cell incubation and processing system for Cytocentric® control of temperature, oxygen, and CO2 levels. Its modular design encloses third-party equipment for full-time control of cell environments during all cell processing. operations.

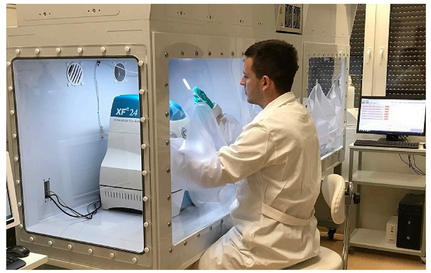
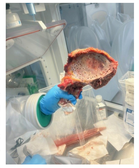


Figure 2: Isolation of Human Bone Marrow Cells Under Controlled Conditions
Human pelvic bones from two different donors, both under the age of 30, were sourced (fresh) from a licensed organ procurement organization (ConnectLife). They were transported to and imported into the Xvivo System in oxygen-proof heat-sealed mylar bags. Tiny holes were drilled into the bone interior and a Microx 4 needle oxygen probe (PreSens) was used to measure bone interior oxygen levels. More holes were drilled and the bone was perfused with DPBS (Sigma Aldrich) containing DNAse (Stemcell Technologies). The perfusate was collected in a sterile bag held open with a custom bag holder. The perfusate was subjected to discontinuous gradient centrifugation (Ficoll). Mononuclear cells were counted. Some were cryopreserved and some were placed into culture for plastic adherence selection.



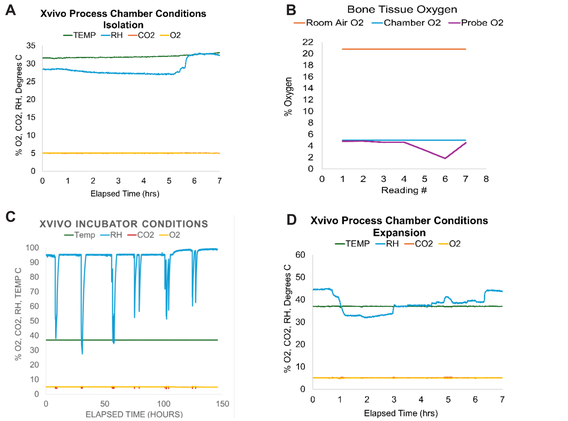


Figure 3: Stable Environmental Conditions Maintained During Handling of Human Bone Marrow-Derived MSCs Prevented Exposure of Cells to Room Air Oxygen
Utilizing the Xvivo System, conditions were controlled to physioxia for bone marrow, 5% O2 plus 5% CO2 in the processing chamber during all isolation steps including centrifugation (A). Bone marrow O2 was below 5%, confirming that room air oxygen had not permeated the bone before cell isolation (B). For cell expansion, the Xvivo System incubator (C) and processing chamber (D) was maintained at constant physioxia (typical week shown). Only changes in relative humidity (RH) were seen with incubator opening and liquid handling events.

Results

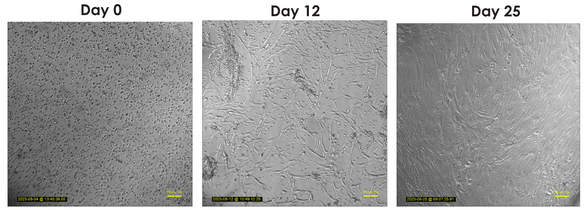


Figure 4: Cell Appearance
Bone marrow MNC were adhered and dividing well by Day 12 of culture. By Day 25, cultures were primarily MSC-like in morphological features.

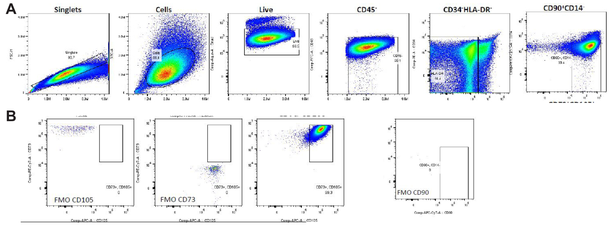


Figure 5: Adherent Bone Marrow Cell Phenotype Consistent with MSC
Flow cytometric analysis was done with a Cytek Aurora and the indicated gating pathway (A) using FMO controls (B). Representative results are shown. The cells were highly autofluorescent, but the bulk of the cells were confirmed as CD45-CD34-HLA-DR-CD14-CD90+CD105 + CD73 +, (average 79.9% +/- 9%) (n = 4, 2 separate cultures from 2 separate donors, 2 different passages each) consistent with an MSC phenotype.

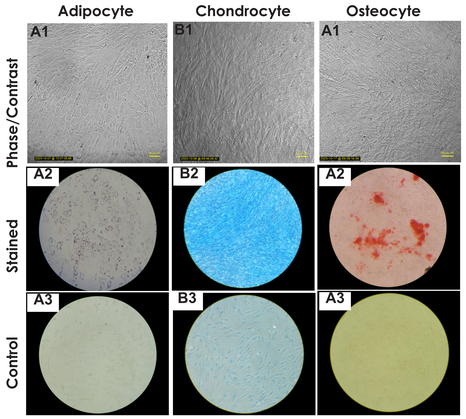


Figure 6: Differentiation of Plastic-Adherent Human Bone Marrow Mononuclear Cells
The MSC were cultured in media (StemPro) for 28 days to force differentiation into adipocyte, chondrocyte, and osteocyte phenotypes. Morphology (black/white photos, and staining with Oil Red O for fat (adipocytes), Alcian Blue for sulfated proteoglycan (chondrocytes), and Alizarin Red for calcium deposits (osteo-cytes), indicated successful differentiation of the MSC into characteristic mature cell types. Representative images from 2 separate experiments with 2 cultures (2 separate donors) are shown.

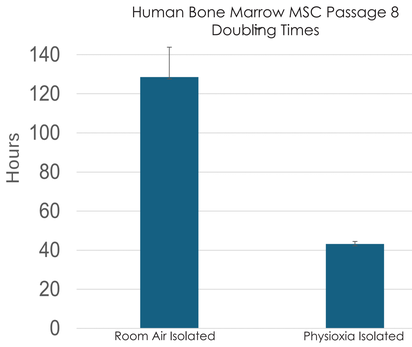


Figure 7: Better Cell Growth in Cells Isolated Under Physioxia
Cells isolated and expanded under unbroken physioxia (O2 5%) had lower doubling times than human bone marrow MSC from a commercial source (Lonza) isolated under traditional room air handling and incubation. After acquiring the cells (frozen) at Passage 2, the Room Air Isolated MSC were thawed and cultured at unbroken 5% O2/ 5% CO2. Even at Passage 8, the MSC isolated under controlled physioxia had lower doubling times (room air, n=3 separate cultures; physioxia, n = 3 separate cultures grown in 3 different cell culture media). This disproved the null hypothesis.

What Does It Mean?

Conclusions:

- **Human bone marrow MNC were isolated without exposing the cells to room air oxygen at any point.**
- **Plastic-adherent MNC expanded under constant physioxia had phenotype and differentiation potential characteristics consistent with bone marrow MSC**
- **Human bone marrow MSC isolated and grown under constant physioxia had more robust growth than those isolated under traditional room air cell culture conditions (BSC and cell culture incubator), even under the same cell expansion conditions.**

Scientific Impact:

- **The artifact that is introduced by room air oxygen into MSC-based assays cultured under traditional conditions may be avoided by protecting MSC from room air.**
- **Protecting MSC from supraphysiologic oxygen during cell isolation can help produce a more robust cell line that can help improve the reproducibility and translatability of MSC-based *in vitro* assays for toxicology.**

References

1. Mantel, C.R., et al., Enhancing Hematopoietic Stem Cell Transplantation Efficacy by Mitigating Oxygen Shock. Cell, 2015. 161(7): p. 1553-65.
2. Broxmeyer, H.E., et al., 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 ex vivo. Curr Opin Hematol, 2015. 22(4): p. 273-8.
3. Henn, A.D., et al., Cell culture oxygen and signaling: Supraphysiologic in vitro oxygen signals through the HIF-CXCR4 pathway in human B cells. The Journal of Immunology, 2017. 198(1 Supplement): p. 143.3-143.3.
4. Kumar, B., et al., Tumor collection/processing under physioxia uncovers highly relevant signaling networks and drug sensitivity. Sci Adv, 2022. 8(2): p. eabh3375.
5. Adebayo, A.K. and H. Nakshatri, Modeling preclinical cancer studies under physioxia to enhance clinical translation. Cancer Res, 2022..