Oxygen Stress in Non-Physiologic Room Air Conditions for Cell Incubation and Handling Attenuates the In Vitro Growth of Human Mesenchymal Stem Cells through Effects on Individual Cell Fate

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Abstract

In vitro assays hold the promise of reducing or replacing animals for routine toxicologic testing. Critical to *in vitro* reproducibility are well-controlled conditions for cell incubation and handling. Previous work by other groups has shown that several cell types including Mesenchymal Stem/Stromal Cells (MSC) suffer tremendous oxidative stress under traditional room air conditions which are hyperoxic (20% O_2) compared to *in vivo* conditions. We previously published that full-time unbroken incubation and handling conditions with full-time physiologically-relevant oxygen levels (5% O_2) can boost MSC growth and keep cells growing longer than oxygen control for incubation only. Our hypothesis for this study is that traditional room air cell culture incubation and cell handling (20% O_2/0.05% CO2) would also affect MSC fate. Human MSC cultures were split into different conditions; room air conditions (traditional CO_2 incubation/HEPA filtered room air handling) or full-time physiologic O_2 and CO_2 (the Xivo System X3). The CytoSMART live cell imaging system was used for time-lapse photography. All cell culture media were pre-equilibrated to the matching cell handling conditions before use. Cell images were recorded every 15 minues over 3-4 days of culture, including early and late stage passages, and analyzed frame-by-frame. Individual cells were labeled and traced for cell fate decisions. Exposure to room air conditions did not affect how much time it took MSC to adhere after trypsinization or start dividing, but did affect how many cells were able to undergo cell division at all. More cells exited the cell cycle and ceased cell division when cultured and handled in room air than under full-time physiologic oxygen conditions. Cell viability for all cultures was greater than 90% at all times. This resulted in an overall cytostatic effect, rather than a cytotoxic effect, that was not obvious unless direct comparisons were made. We concluded that full-time protection of human MSC from room air, during cell incubation as well as cell handling, helped keep individual cells in the cell cycle to produce the observed population-wide effects.

Background

- MSC are isolated from tissues that thrive at oxygen levels much lower than room air (0-5% O2)
- Traditional room air cultured MSC behave quite differently from MSC in vivo [1]
- Toxicologic assays experience oxygen artifact when performed in room air [2]
- Room Air O_2 levels are highly stressful for human MSC[3]
- Previous studies showed higher cell yields when cells were cultured under full-time 5% O2 rather than incubated in part-time control (incubated at 5% O2 and handled in a room air)

Objectives

Determine source of change in behavior of MSC when cultured in room air oxygen. Was there more cell death, a delay in division, reduced generations, or a decrease in % cells dividing in:

A. Traditional room air CO_2 incubator conditions (20% O_2/5% CO_2) and room air handling vs.

B. Full-time physiologic temperature, O_2 and CO_2 for all cell incubation and handling (in the Xvivo System X3)

References

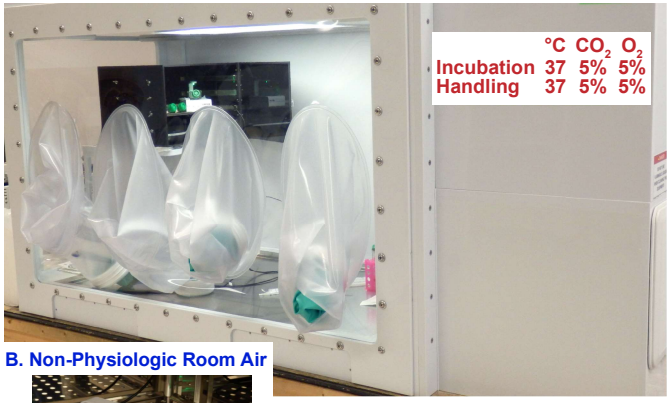
1. Marfy-Smith, S.J. and C.E. Clarkin, Are Mesenchymal Stem Cells So Bloody Great After All? STEM CELLS Translational Medicine, 2017. 6(1): p. 3-6.
2. Kumar, A., et al., Quantifying the magnitude of the oxygen artefact inherent in culturing airway cells under atmospheric oxygen versus physiological levels. FEBS Lett, 2016. 590(2): p. 258-69.
3. Banegowda, S.V., et al., Atmospheric oxygen inhibits growth and differentiation of marrow-derived mouse mesenchymal stem cells via a p53-dependent mechanism: implications for long-term culture expansion. Stem Cells, 2012. 30(6): p. 975-87.

Conflict of Interest Disclosure

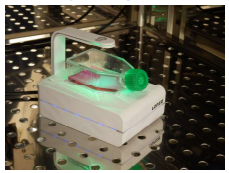
The authors all are or have been employees of BioSpherix, Ltd.

Experimental Design

A. Full-Time Physiologic Atmosphere



B. Non-Physiologic Room Air



	°C	CO_2	O_2
Incubation	37	5%	16-19%
Handling	25	0.05%	20%

Figure 1. Experimental Set-Up. Human bone marrow MSC (Lonza, Gmb) were thawed under Full-Time Optimal Conditions (37°C, 5%CO2, 5%O2) and immediately split into three flasks for each condition. One set was removed to a standard external CO_2 incubator for Suboptimal Room Air conditions. One flask of each set was used for continuous monitoring with the Lonza CytoSMART microscope and two flasks were used to pool with for cell counts at each passage. MSC were monitored for density and the subcultured at < 80% confluent. Medium was changed every 3-4 days with or without passaging. All materials were equilibrated to the proper gas mixture and temperature before use. Cells were trypsinized, washed, and counted at each passage. Cell coverage data and time-lapse photomicrographs were provided by CytoSMART. Images were analyzed for timing of mitotic events and individual cell fate analyses.

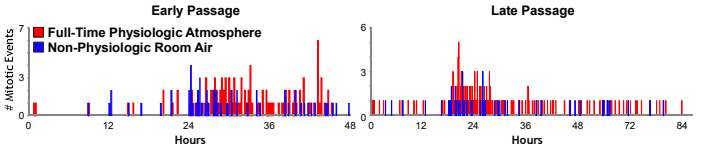
Results

	Passage 4							Passage 7						
	Total Hours	Adherent By (hrs)	# Cells migrated	Starting Cell #	Ending Cell #	Fold Increase	% viable	Total Hours	Adherent By (hrs)	# Cells migrated	Starting Cell #	Ending Cell #	Fold Increase	% viable
Physiologic	45	4	21	132	224	1.7	93	84	5.3	21	132	224	1.7	93
Room Air	45	6	29	123	176	1.4	96	84	6.8	29	123	176	1.4	96

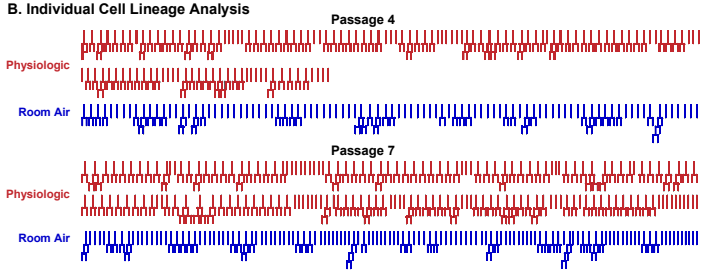
Table 1. MSC Growth Characteristics. Cell Death Does Not Explain Fewer MSC without Full-Time Oxygen Control. Grids were superimposed on each frame for individual cell fate analysis. After each individual cell movement and mitotic events were recorded, the start and end cell numbers in the field were recorded. The number of cells that migrated from their starting box were recorded. Cell viability was measured after trypsinzation from the flasks by trypan blue exclusion.

Results (Continued)

2A. Population Dynamics - Timing



B. Individual Cell Lineage Analysis



C. Clonal Capacity

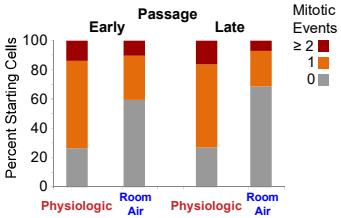


Figure 2. Room Air Culture Reduces the Number of MSC by Reducing the Number of Cells Actively Dividing

(A) Frame-by-frame cell fate analysis of two sets of time-lapse videos recording mitotic events per 15 mins over 2-4 days of undisturbed culture (at Passage 4 or 7) showed few mitotic events before a burst of cell division at about 24 hours in both conditions so there was no major delay in initiation of cell division after trypsinization in room air cultured cells. (B) Each line represents an individual cell, and each "Y", a cell division. The MSC exposed to room air exhibited fewer mitotic events. A few room air cells were able to divide three times, but most cells did not divide at all. In late passage, cells cultured and handled under controlled oxygen conditions were less likely to senesce. (C) A graph showing that MSC were about twice as likely to exit the cell cycle and never divide again if cultured in traditional room air conditions for incubation and handling.

Conclusions

Traditional non-physiologic cell culture and handling conditions have a dramatic effect on cell behavior

Full-Time physiologic oxygen control protected the ability of individual cells to remain in the cell cycle and not senesce