BioResearch

Full-time Control of Conditions During Cell Handling Enhances the Growth of Human Mesenchymal Stromal Cell Cultures

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Introduction

The long-term goal of any *in vitro* research effort is to translate findings into *in vivo* therapeutics. However, *in vitro* conditions for incubating and handling cells are often far from *in vivo* conditions. Human mesenchymal stem/stromal cells (MSC) are in clinical trials as a cellular therapy for dozens of different disease states. Therefore, optimization of MSC *in vitro* expansion and function is critical.[1]

Harvested from tissues that normally reside at oxygen levels far lower than room air, like bone marrow, umbilical cord, and adipose tissue, MSC are subjected to oxidative stress in supraphysiologic room air oxygen conditions (~20% O_2).[2] Within 5 minutes of room air oxygen exposure, protein-level changes occur in Hypoxia-Induced Factor 1 (HIF-1) proteins, the master oxygen response regulators that affect cell growth and differentiation.[3] This suggests that routine cell incubation and cell handling in room air may affect MSC expansion *in vitro*.

Here, we compared the growth of MSC culture in traditional room air conditions (no oxygen control during incubation or handling) with full-time optimal (5% O_2) conditions. Since many researchers seeking physiologically relevant oxygen conditions (physioxia) often perform cell handling in a traditional biological safety cabinet (BSC) with HEPA-filtered room air, interrupting physioxic conditions with brief room air exposures, we went on to also assess the effect of these interruptions in optimal conditions on MSC expansion.

Materials and Methods

Two long-term studies were performed. In each, Lonza Poietics™ Human Bone Marrow MSC were thawed and split into six identical cultures in Lonza hMSC Basal Medium plus MSCGM™ hMSC SingleQuots™ Kit. Lonza Trypsin/EDTA for MSC, and Lonza DPBS w/o Ca++/Mg++ were used for subculturing. Cellular growth was monitored using cell coverage data generated by the CytoSMART™ System (Lonza) as well as microscopic cell counts with trypan blue (Sigma, St. Louis, MO, USA) for cell viability at each passage. To capture the cell population expansion, at each passage, cumulative cell numbers were calculated by summing total calculated viable cell populations with previous cell passage numbers minus cells used for the new passage. Before use, cell culture media were warmed and pre-equilibrated to the appropriate cell handling gas levels. Cell culture medium oxygen levels were measured using an immersion oxygen probe sealed through the wall of a T-flask.

MSC cultures were split into two different conditions. One set of three cultures were incubated in the Xvivo System X3 (BioSpherix Ltd., Parish, NY, USA) for full-time optimal conditions (5% O_2, 5% CO_2) for cell incubation and handling (Figure 1A). Three other cultures were housed within a traditional, suboptimal room air incubator. All cell handling of this culture group was in traditional (20% O_2, 0.05% CO_2) HEPA-filtered room air conditions. In both conditions, one flask was incubated on a CytoSMART™ System for time-lapse microscopic image-based monitoring (Figure 1B).

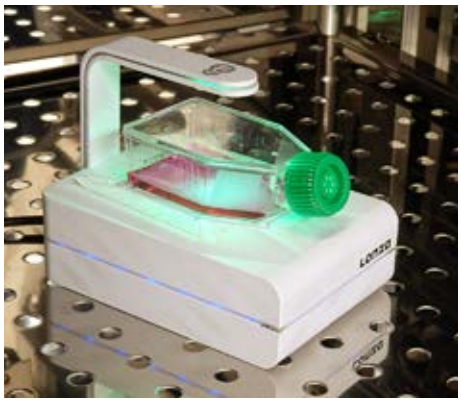
Although areas of bone marrow naturally have extremely low oxygen tensions (0.6–2.8% O_2) due to local physiology[4], the higher end of the *in vivo* oxygen range for bone marrow intravascular space (5% O_2) was chosen for this study. This was to avoid pericellular oxygen depletion due to increasing oxygen consumption rates by growing cell populations during the study.

To examine the effects of just sub-optimal cell handling on MSC grown in physioxic cell incubation, a second study was performed. Here, the growth of MSC cultures handled and incubated under full-time physioxic conditions in the Xvivo System X3 (Figure 1A) was compared with MSC handled in traditional room air BSC conditions (20–25 minutes), but incubated under physiologic oxygen in an incubator subchamber (5% O_2, 5% CO_2) (C-Chamber with ProOx C21 O_2/CO_2 gas controller, BioSpherix) (Figure 1C).

1A Full-time Optimal Conditions

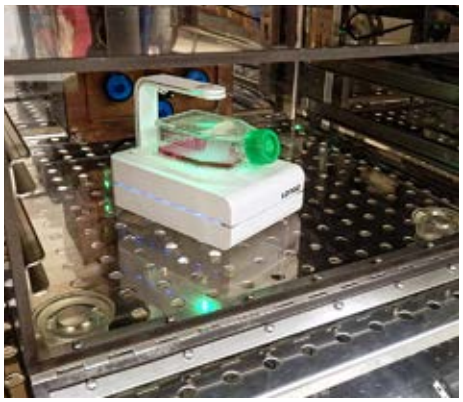
	°C	CO_2	O_2
Incubator	37°C	5%	5%
Handling	37°C	5%	5%

1B Traditional Sub-optimal Conditions



	°C	CO_2	O_2
Incubator	37°C	5%	16–19%
Handling (BSC)	25°C	0.05%	20%

1C Part-time Optimal Conditions



	°C	CO_2	O_2
Incubator	37°C	5%	5%
Handling (BSC)	25°C	0.05%	20%

Figure 1

Experimental set-up. Cells were thawed under physioxic conditions (37°C, 5% O_2, 5% CO_2) and immediately split into two conditions for each study, either **(A)** full-time control of physioxic conditions for cell incubation and handling in the Xvivo System model X3 and **(B)** traditional conditions with no oxygen control, or **(A)** full-time optimal conditions and **(C)** part-time optimal conditions with an oxygen controlled sub-chamber for incubation, but no oxygen control (traditional) cell handling. The CytoSMART™ System monitored cells without disturbing the cultures during incubation in each setting.

Results

Full-time Optimal Conditions for Routine Cell Handling as well as Incubation Improve Human MSC Yields

Since MSC originate from tissues with naturally low oxygen levels, we tested physiologically relevant oxygen levels for human MSC expansion *in vitro*. Cell yields under full-time optimal conditions (5% O_2, 5% CO_2 for all incubation and handling) were greater than cell yields under traditional open room air conditions (no attempt to control oxygen levels). Faster, more robust MSC culture expansion was seen in cultures under full-time optimal conditions as determined by CytoSMART™ Cell Coverage Data in early, mid-, and late passages (Figures 2A, B, and C) as well as by overall cumulative cell numbers (Figure 2D).

Interrupting Optimal Conditions for Room Air Cell Handling Reduces MSC Yields

A common practice in many labs trying to emulate physiologic conditions *in vitro* is to incubate the cells in an oxygen-controlled incubator, but handle the cells in room air in a HEPA-filtered room air BSC. Since HIF-alpha levels can be modified so rapidly by supraphysiologic oxygen levels, we looked more closely at just the effect of room air exposure during room air BSC cell handling.

We compared the effects of full-time optimal conditions for incubation and handling in the Xvivo System X3, with part-time optimal conditions in which MSC were incubated in an oxygen-controlled incubator subchamber, but moved to room-air BSC conditions for cell handling. We found that under part-time optimal conditions, MSC growth was impaired as compared to full-time optimal conditions (Figure 3). Cell coverage data show population growth differences in early- (Figure 3A), mid- (Figure 3B), and late- (Figure 3C) cell passages. Overall cumulative cell counts showed these differences as well as earlier culture senescence in MSC exposed to room air during handling (Figure 3D).

Room Air Exposure Produces a Non-obvious Cytostatic Effect

Cell viability was greater than 90% at all times in these cultures (inset Figures 2D, 3D), so no obvious cytotoxicity was seen. The effect was cytostatic. Unless direct comparisons were made between cell handling conditions, it would not be obvious that the cells were being negatively affected by room air exposure.

Cell Culture Medium is Out of Optimum for Long Periods of Time During Equilibration with the Incubator

Looking into why such seemingly brief exposures to sub-optimal conditions during routine cell handling affect MSC growth so dramatically, we looked at cell culture oxygen levels in cell culture media after cell handling. We placed a T-25 flask with 5 mL of cell culture medium equilibrated to 20% or 5% O_2 in an incubator and monitored medium oxygen equilibration with incubator or chamber oxygen levels. It took over 2 hours after the flask was placed in the incubator for the medium to get close to 5% O_2 and over 4 hours for the medium to fully equilibrate (Figure 4). This implies that cells are out of optimum for long periods of time when subjected to even brief cell handling in sub-optimum conditions. Long equilibration times, along with the rapid changes in HIF-1 levels, may help explain why such brief interruptions in optimal conditions had such long-lasting effects on cell growth. It also shows that pre-equilibrating cell culture media to optimal gas levels before use can reduce time that cells are subjected to suboptimal conditions.

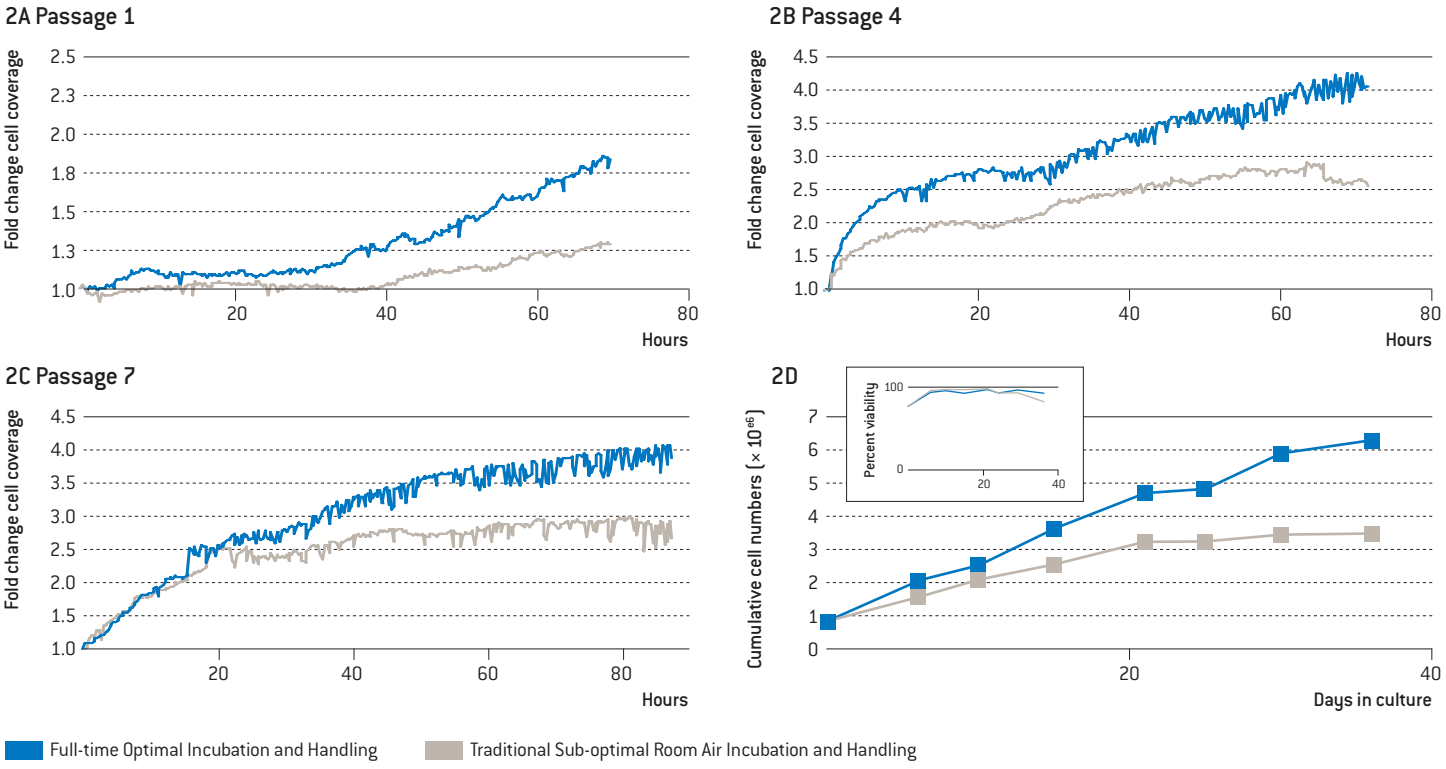


Figure 2

MSC expansion in full-time optimal conditions is superior to cell growth in traditional sub-optimal room air conditions. A traditional CO_2 incubator for incubation and HEPA-filtered room air conditions for cell handling did not yield as much cell growth as cells grown under full-time optimal conditions. CytoSMART™ Cell Coverage Data from early-, mid-, and late-passage cells (Panels A, B, and C) and overall cell yields by manual cell counts (Panel D), all showed this effect. Cell viability was high for both conditions (inset), but started to decrease in the last passage of the cells that were incubated and handled in room air conditions (traditional). So reduced cell yields were an effect that would go unnoticed if a direct comparison was not made. A paired T-test (2-tailed) showed unlikely statistical overlap between the groups at each passage ($p = 0.0079$).

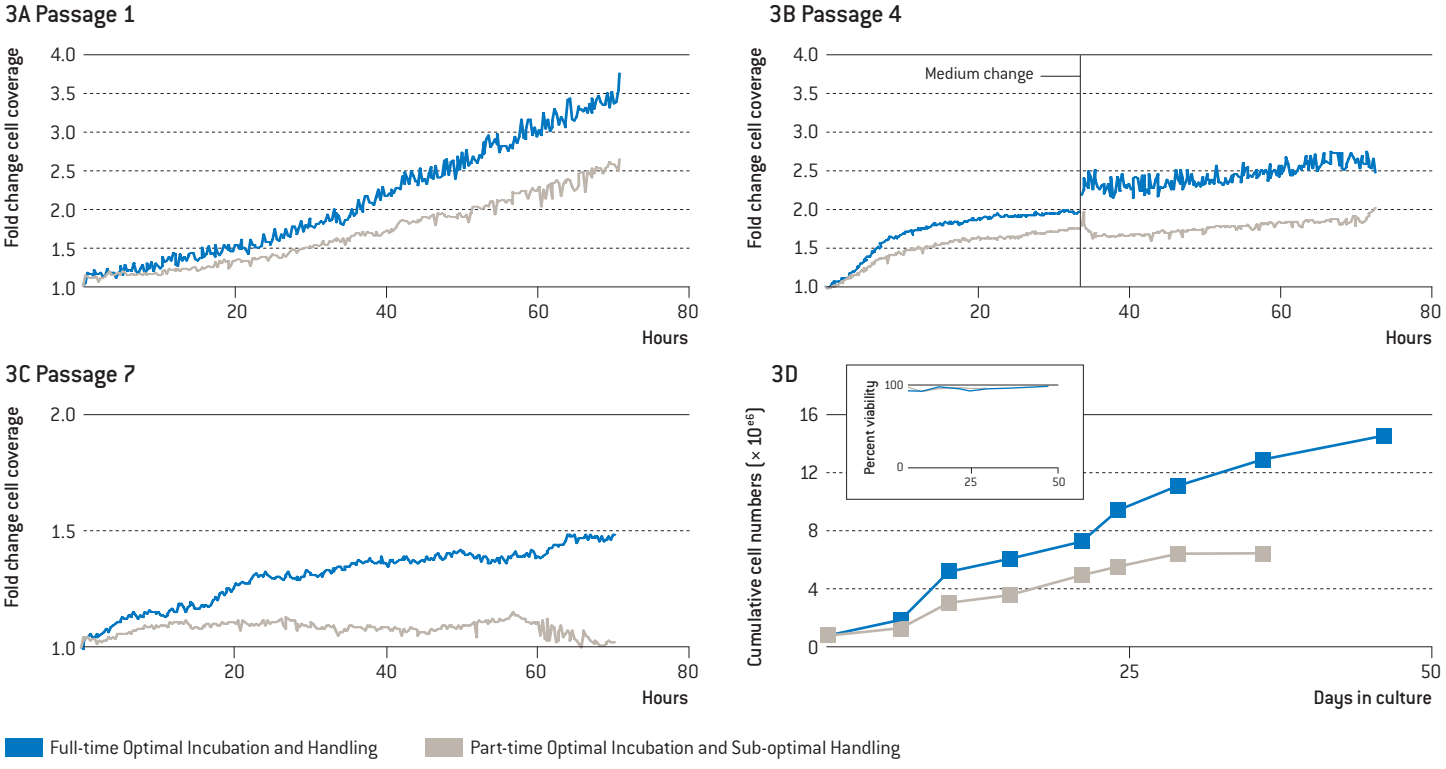


Figure 3

Interruption of full-time optimal cell culture conditions reduces MSC yields. Under full-time optimal conditions for both incubation and handling, human MSC grew to higher yields as compared to cells exposed to room air conditions for routine passage. This was found in early-, mid-, and late-passage cultures, as assessed by CytoSMART™ Cell Coverage (Panels A, B, and C) and by trypan blue cell counts (Panel D). Cells in both conditions exhibited high live cell percentages (inset), suggesting a cytostatic effect rather than a cytotoxic effect. Higher overall cell expansion was found in cells under full-time optimal conditions as well as more passages. A 2-tailed paired T-test showed unlikely statistical overlap of cell yields at each passage ($p = 0.0072$).

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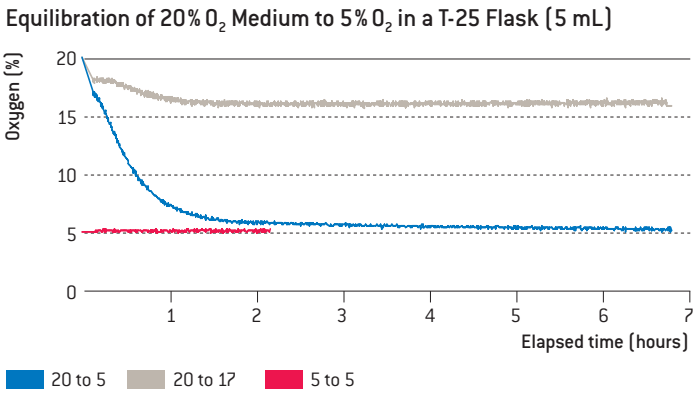

Figure 4

Oxygen equilibration dynamics of cell culture medium. Oxygen dissolves poorly in aqueous solutions. When medium sits undisturbed in a cell culture incubator, it takes time for the medium to equilibrate with the oxygen of the environment. It was well over an hour for medium equilibrated to room air (20% O_2) to completely equilibrate to the 5% O_2 levels in the incubator (gray line). During this time, even though the cells were placed in the incubator, seemingly at the proper oxygen levels, the cells were actually out of optimum conditions. Cell culture medium equilibrated to traditional CO_2/room air incubator levels (~17% O_2) in about 2 hours from room air oxygen levels (blue line). Medium pre-equilibrated to 5% O_2 had no effective equilibration time that could affect cells (red line). This could help explain why even brief exposures to room air conditions had such long-lasting effects on cell cultures.

Conclusions

Undisturbed observations of human MSC growth in unbroken cell culture conditions were possible by combining the CytoSMART™ System with the Xvivo System X3. MSC yields were higher in full-time optimal conditions than in traditional room-air conditions. Even brief interruptions in optimal conditions for cell handling negatively affected MSC growth. This was not an obvious cytotoxic effect but a non-obvious cytostatic effect. Cell culture media remained out of optimum for hours while oxygen levels equilibrated with the incubator, which may affect cells longer than is readily apparent.

References

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