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Oxygen: Too Much of a Good Thing

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Oxygen-controlled incubators can provide the low O₂ conditions necessary for stem cells derived from bone marrow and adipose tissue.

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Stem cell research, whether using embryonic or somatic sources, promises to provide not only a better understanding of the fundamental principles of cell differentiation, but also a whole host of life-changing or even life-saving cures.

Although it is still a developing area, scientists are learning just as much about how to culture and use stem cells most effectively as they are about their unique potential. For example, optimizing culturing conditions is essential for maximizing the data obtained from any experiments, as well as the quality of daughter populations and subsequent differentiated cells.

Often overlooked yet important, certain stem cell populations naturally reside in locations with very low oxygen

tensions, such as bone marrow and adipose tissue. This means that when culturing cells from such sources, in addition to monitoring CO₂, O₂ levels should be carefully controlled to the lower end of the physiological O₂ scale to best duplicate natural conditions. The most efficient way of controlling this is through the precise introduction of N₂ into the incubator culturing system.

The following looks at the measurable differences in stem cell maintenance and subsequent differentiation between a standard CO₂ incubator and one capable of controlling O₂ levels.

Oxygen's Importance

Oxygen is one of the most essential elements to life on earth and forms about 21% of the "air" around us. As a result, laboratory cell culture is generally carried out without control over oxygen levels, and this does not always provide the correct O₂ tensions at the level of the cells. This is due to the proximity of the *in vitro* culture to the atmosphere as opposed to the *in vivo* situation where oxygen is transported around the body via the blood and delivered to different cells and tissues at a lower O₂ tension.

In addition, a growing number of research studies have shown that drastically reduced oxygen conditions are favored by a number of stem cell populations, especially those derived from bone marrow and adipose tissue. Traditionally, these lower O₂ levels have been referred to as hypoxia, but it is actually an *in situ* normoxia for the cells and forms part of a scale of O₂ tensions referred to as physiological O₂.

This range covers the *in situ* normoxic conditions for all



The Thermo Scientific Heracell 240i CO₂ incubator with O₂ control (right) has a 240-L (8.5-cu-ft) capacity and is available in a smaller 150-L (5.3-cu-ft) size (left).

cell types, from stem cells to fully differentiated and O_2 saturated cells. Therefore, to recreate this physiological O_2 range in an incubator, the chamber O_2 must be in the range of 2% to 10%, depending on the cell type.

Application

We focused on the lower end of this spectrum and looked at the advantages of controlling incubator O_2 levels for stem cells. Low O_2 conditions were shown to improve the overall therapeutic function of bone-marrow-derived mesenchymal stem cells via a variety of biochemical effects. [1]

Key factors such as cMet—the major receptor for hepatocyte growth factor (HGF)—were unregulated, and the Akt signaling pathway was activated, which is important in cell signaling and survival. Stem cells cultured under atmospheric and low O_2 conditions both increased revascularization following ischemic injury, but this occurred much quicker with cells conditioned in the latter environment.

Research also showed that the low O_2 incubator environment improved stem cell self-renewal without reducing their ability to differentiate. [1, 2] Such an environment was even proposed as one of the key factors in the maintenance of undifferentiated stem cells *in vivo*. [2] This research demonstrated that preadipocytes remained undifferentiated in these conditions and noted the involvement of hypoxia-inducible factor-1 (HIF-1) and pef-1, key genes in the inhibition of differentiation.

In addition to HIF-1 stabilization, VEGF and other angiogenic factors were upregulated, which have previously been noted as important to stem cell survival and self-renewal. [3]

Interestingly, research has suggested that the level of O_2 is important in determining the survival of stem cells and subsequent levels of progenitor cells. Work on bone-marrow-derived hematopoietic stem cells showed that cells in increasingly differentiated stages within the hematopoietic cell hierarchy reside in areas with increasing levels of O_2 saturation. [4] Furthermore, the special low O_2 metabolic properties and concomitant self-renewal capabilities have been described as the “stem cell paradigm”. [5]

As a result, being able to accurately control O_2 levels during culturing is essential, not only for work on stem cells but also for subsequent generations of progenitor cells, and even those that have fully differentiated.

Evaluating O_2 control

Although stem cell research in low oxygen environments has been conducted for a number of years, most has been carried out in specialized low O_2 chambers. With such research increasing, modern multi-gas incubators

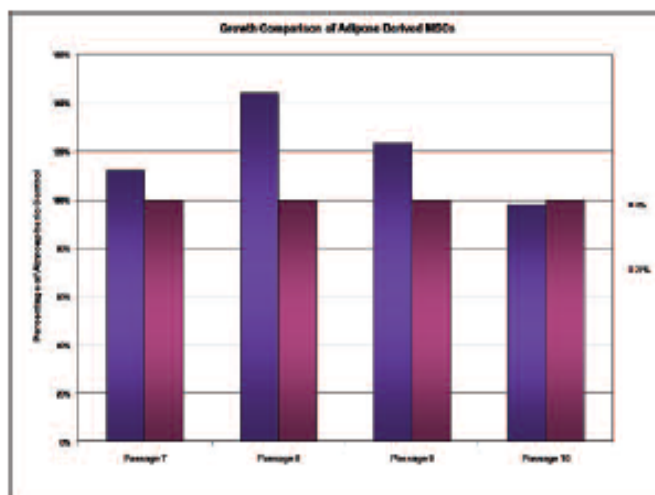


Figure A. Growth comparison of adipose-derived MSCs hypoxic (4% O_2) vs. atmospheric (21% O_2). Viable cell counts expressed as percentage of control. Passage number indicates the number of passages since thaw.

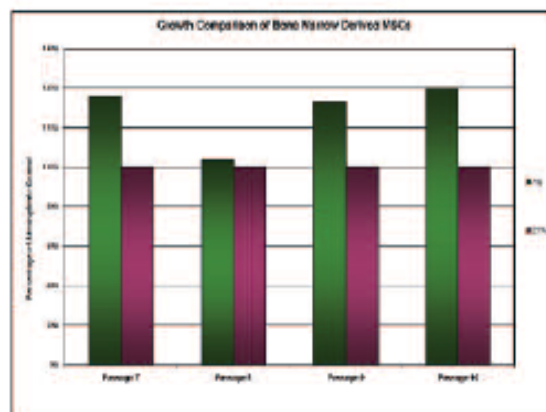


Figure B. Growth comparison of bone-marrow-derived MSCs hypoxic (4% O_2) vs. atmospheric (21% O_2). Viable cell counts expressed as percentage of control. Passage number indicates the number of passages since thaw.

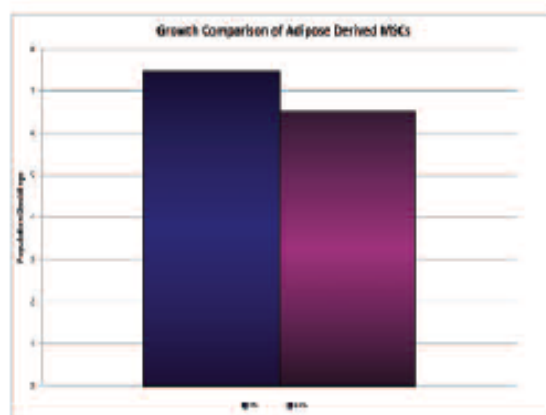


Figure C. Growth comparison of adipose-derived MSCs hypoxic (4% O_2) vs. atmospheric (21% O_2). Total population doublings over 4 passages.

now offer precise control of oxygen, as per the specialized chambers, and combine this with superior capacity and functionality.

The Thermo Scientific Heracell 240i CO₂ incubator features precise oxygen concentration maintenance (between 1% to 21%) using N₂, which is controlled via its interactive touchscreen navigator, iCAN. The incubator also has a built-in high-temperature decontamination system to ensure the chamber is aseptic, which is essential for all cell culture processes.

The Heracell 240i was recently evaluated for culturing stem cells in a low O₂ environment (4% ± 0.2% O₂) using human mesenchymal stem cells from adipose tissue and bone marrow in conjunction with specialized media developed to support stem cell culturing activities.

The data and general observation of the cells showed that the Heracell oxygen-controlled incubator successfully created a stable low O₂ environment suitable for advanced stem cell culture.

The advantages of hypoxic culture conditions became clear early on, as both cell types had greater viable cell counts (Figures A and B).

The HAMSCs began to senesce following passage 9. This senescence is common with MSCs and became apparent sooner in the low O₂ condition, perhaps due to the faster growth of these cells in this condition.

Furthermore, the population doubling data showed a clear advantage for the hypoxic environment (Figures C and D).

When cultured for 18 days to differentiate into adipocytes, the HAMSCs achieved double the viable cell yield under hypoxic conditions compared to normoxic (Figure E).

Some differentiation was observed with the HAMSCs, as demonstrated by the colonies of red stained adipocytes (Figure F). This differentiation was much less than would normally be expected, even in the control condition. Our experience indicates that this is likely due to the age (passage 11) of the cultures. Little or no differentiation was observed in all of the adipose-derived MSC conditions, which again is likely due to the age of the cultures. The data also shows that growing cells in standard conditions and differentiating the cells under hypoxic conditions seems to provide no benefit.

Discussion

Routine cell culture requires that cells are incubated at 37 °C and 5% CO₂. A number of research studies though have shown that, when human stem cells are cultured at 37 °C, 5% CO₂ and ≤ 4% O₂ (low O₂ conditions), the effect prolongs proliferation/self-renewal and yields more

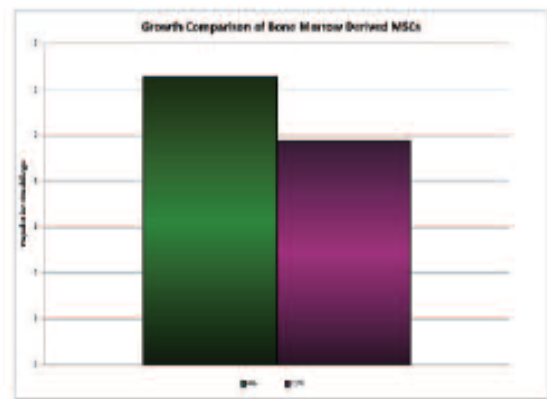


Figure D. Growth comparison of bone-marrow-derived MSCs hypoxic (4% O₂) vs. atmospheric (21% O₂). Total population doublings over 4 passages.

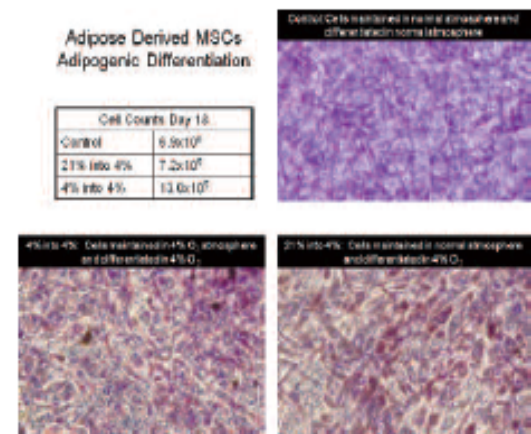


Figure E. Characterization of adipogenic differentiation characterization by Oil Red O staining and viable cell counts – bone-marrow MSCs.

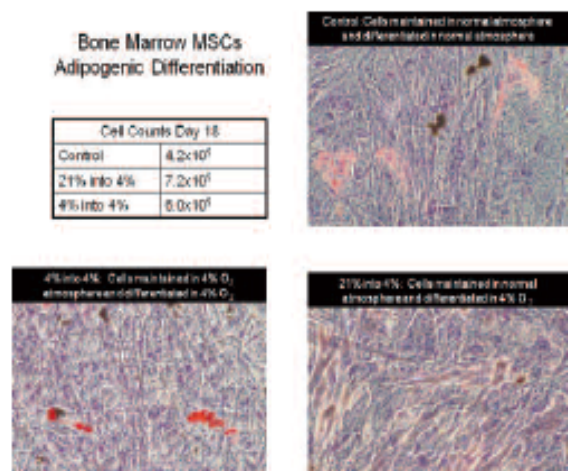


Figure F. Characterization of adipogenic differentiation characterization by Oil Red O staining and viable cell counts – adipose-derived MSCs.

cells when the cultures are allowed to differentiate.

In this experiment, the Thermo Scientific Heracell 240i CO₂ incubator with O₂ control simulated a low O₂ environment and was compared to incubation under atmospheric conditions in a standard CO₂ incubator. The results align with published results and demonstrate that this instrument can provide the correct environment for moving stem cell research forward.

Furthermore, the highly precise maintenance of the defined conditions and the flexibility of the incubator ensure that it is also suited for studying the hierarchy of cell lines, from stem cells to fully differentiated cells.

For more information, please contact Douglas Wernerspach, Global Product Manager, CO₂ Incubation, Thermo Fisher Scientific, at Douglas.wernerspach@thermofisher.com.

Materials:

Cells

- CET Human Adipose-Derived Mesenchymal Stem Cells - HAMSC (Thermo Scientific). These are isolated from human red bone marrow collected via a bone marrow aspiration procedure. HBMSCs may be differentiated into adipogenic, chondrogenic, osteogenic, neuron-like cells and other cell types.
- CET Human Bone Marrow Mesenchymal Stem Cells - HBMSC (Thermo Scientific). These are isolated and expanded from human lipoaspirate using enzymatic treatment. HAMSCs are capable of being grown in large numbers and have multilineage differentiation potential (including osteocytes, adipocytes and neuron-like cells), as well as immunomodulatory functions in animal models.

Media

- Growth media - HyClone AdvanceSTEM™ Mesenchymal Basal Medium with 10% HyClone AdvanceSTEM™ Growth Supplement (Thermo Scientific). This media has been developed to provide consistent support and growth conditions for all mesenchymal stem cell populations.
- Differentiation Media - HyClone AdvanceSTEM™ Adipogenic Differentiation Kit (Thermo Scientific). This media has been developed to support the differentiation of a variety of human mesenchymal stem cells, including HAMSCs and HBMSCs, into adipocytes.

*Part of original content not featured online.

References

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4. Cipolleschi, M.G., P. Dello Sbarba, and M. Olivetto. 1993. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* 82: 2031-2037.
5. Ivanovic, Z. 2009. Hypoxia or in situ normoxia: The stem cell paradigm. *J. Cell. Physiol* 219: 271-275.

Control growth condition:

Incubator: Standard laboratory CO₂ incubator
Settings: 5% (± 0.1%) CO₂; 21% (ambient) O₂

Experimental growth condition:

Incubator: Thermo Scientific Heracell 240i CO₂ incubator with O₂ control
Settings: 5% (± 0.1%) CO₂; 4% (± 0.2%) O₂

Methods:

- 1) HAMSCs and HBMSCs (passage 6) were seeded at 1x10⁵ cells per T-25 flask in mesenchymal expansion media (AdvanceSTEM HyClone) + 10% supplement for proliferative culture.
- 2) Cells were incubated at 37°C either in a normoxic or hypoxic (Heracell 240i) environment (as per the conditions detail above).
- 3) After 4 days, or 90% confluency, cells were harvested using HyQase, and cell count and viability were recorded using HyQ Trypan blue.
- 4) Cells were re-seeded at 1x10⁵ cells per T-25 flask.
- 5) Steps 2-4 were repeated until passage 10 when cells were set up for differentiation into adipose cells - cultured in Adipogenic Differentiation Media (AdvanceSTEM HyClone) + 10% supplement.
- 6) After 18 days of differentiation, the cells were fixed and stained with Oil Red O and a final cell count was performed.

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