by Mary Kay Bates

Reprinted from *American Laboratory* Web Exclusive October 2012

Culturing Cells Under Hypoxic Conditions for Biologically Relevant Results

For many decades, animal cells have been cultured in air supplemented with carbon dioxide, but new applications for cell therapies require conditions mimicking those in vivo. In the body, oxygen concentrations range from 1 to 12%, rather than the 21% in the atmosphere. Cells cultured in low oxygen, or hypoxia, grow faster, live longer, and show lower stress. A cell culture incubator that provides nitrogen gas in addition to carbon dioxide is the best way to achieve hypoxic conditions.

Oxygen and the cellular process

Take a deep breath. Feel your diaphragm and abdomen expand. Let it out. You just fed oxygen to all of your tissues (and carried away carbon dioxide waste), and you do it constantly without thinking about it.

Obviously, all cells need oxygen to function and survive. And it turns out that oxygen itself regulates nearly every cellular process, from cell metabolism, to differentiation, to cell division. In the laboratory, we commonly grow cells in air supplemented with 5–10% carbon dioxide gas to complement the sodium bicarbonate buffer in the growth medium, which balances the pH. Since we breathe air, taking it into our bodies through our lungs and bloodstream, it makes sense to culture cells in vitro in the same air that we breathe. Or does it?

Oxygen concentration

It turns out that inside our bodies, within the tissues, oxygen concentrations are much lower than the 20–21% oxygen that is in the atmosphere. In fact, oxygen in arterial blood is only about 12%. Deeper in the tissues, oxygen concentrations vary, depending on distance from arterial blood vessels. For example, oxygen ranges from 0.5 to 7% in the brain; 1–5% in the eyes; 4–12% in the liver, heart, and kidneys; and 3–5% in the uterus.[1,2] Clearly, oxygen concentrations in the body are much lower than in the atmospheric air.

Historical perspective on cell and tissue culture

Cell lines have grown quite happily for decades in atmospheric conditions supplemented with carbon dioxide gas. When cell culture was in its infancy, cells were grown in glass dishes on the benchtop. Most cell lines, beginning with HeLa, were derived from aggressive cancers that quickly adapted to new, less than ideal conditions. But as culture skills developed, as understanding grew, as tissue culture expanded to more laboratories, conditions for cells also improved. Time and testing brought new advancements, including better growth media and supplements; humidified incubators heated to body temperature, CO_2 gas to carefully maintain the proper pH, and so on. Along with developing and fine-tuning culture media, researchers sometimes considered the contributions from oxygen as well.

Alan Richter and colleagues in 1972[3] first noted that culturing cells in low oxygen increased the plating efficiency or the number of cells that successfully adapted to growing in a plastic dish. In 1977, Packer and Fuehr[4] demonstrated that culturing in 10% oxygen dramatically increased the lifespan of human fibroblasts, extending it by 25% compared to the lifespan of cells grown in "normal" 20% oxygen. Over the succeeding years, researchers continued to learn more about the biology of physiological oxygen concentration and how it affects cells, including on the molecular level. But in general, low oxygen, or hypoxic, culturing has not received much attention, and many cell culturists are not even aware of this approach.

Advanced technologies using cultured cells

In the twenty-first century, cell culture has come of age. Many advanced applications are now routine, including culturing human embryos for in vitro fertilization (IVF), nurturing a few simple human tissues such as replacement skin for burn victims, and growth of new blood vessels and bladders. Research is continually pushing frontiers for cell-based therapies such as stem cell treatments and growth of replacement organs. Personalized medicines produced from cultured cells are also advancing rapidly; some, such as monoclonal antibodies for cancer and autoimmune treatments, are already used in the clinic. For pharmaceutical and cosmetic manufacturers, cultured cells can reduce use of animal testing. When these products are to be used in humans, it is vital to use culture conditions that will best predict responses in vivo.

These revolutionary techniques—using cultured cells for human therapy—mean that more attention is being paid to better mimic conditions in the body, including providing oxygen at physiological levels.

Use of hypoxic conditions to yield better results

In the last 10–15 years, many researchers have demonstrated that culturing different cell types in low oxygen provides myriad beneficial results. For example, when primary mouse embryonic fibroblasts (MEFs) were cultured in 3% oxygen instead of 20% oxygen, the senescence that commonly occurs after about 28 days of growth was avoided[5] (see *Figure 1*). In addition, the cells in lower oxygen grew faster, showed less DNA damage, and had fewer stress responses.

A different group showed that immune cells cultured at low oxygen behaved as though they were in a healthy body, but the same cells cultured at atmospheric oxygen sent signals as though they were fighting off an infection.[6] Thus, culturing cells under oxygen conditions that are "normal" for cells in the body makes the cells grow faster, healthier, less stressed, and with less DNA damage.

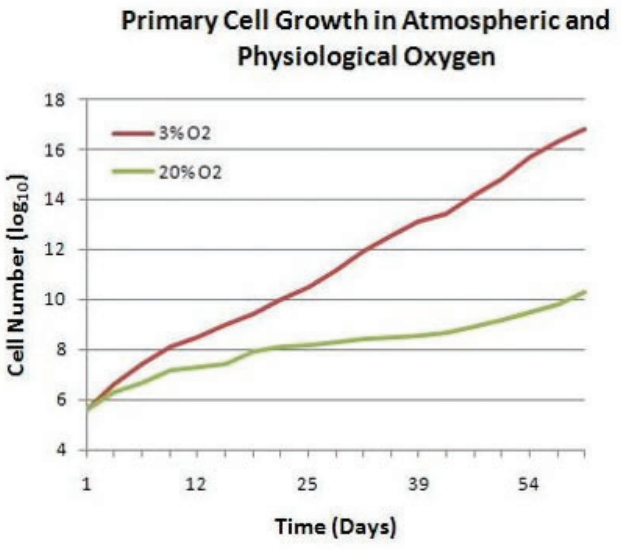

Figure 1 - Primary cell growth in atmospheric and physiological oxygen.

Stem cells are routinely cultured in low oxygen.[7] Many scientists find that culturing them at low oxygen is critical to maintain their normal stem cell characteristics and to keep them from differentiating. Recently, Lengner et al.[8] showed that human embryonic stem cells (hESC) must be cultured in 5% oxygen to retain their pluripotency. Culturing these cells at atmospheric (20%) oxygen caused chromosomal aberrations. In concert with these findings, IVF clinics commonly culture human embryos at 3–5% oxygen to ensure proper development.

Established cell lines and tumors

While most hypoxia research has focused on the most physiologically relevant cells, i.e., primary cells and stem cells, Richter also found that neoplastic cells grew better in low oxygen.[3] As early as 1958, suspension cells were found to grow better in low oxygen.[9] Long-established cell lines—including K562,[10] MCF7, and A549[11]—are affected by hypoxic growth. In humans, solid tumors are well known to have hypoxic centers that are not perfused by the bloodstream. These areas contain necrotic cells that are resistant to chemotherapy and radiation.[12] Thus, it makes sense to use hypoxic culture for cells used in cancer models, in search of new therapies or testing drug effectiveness and toxicities.

Options for hypoxic cell culture

There are currently a few ways to generate hypoxic conditions for cultured cells. One involves using modular gas chambers inside a standard CO_2 incubator. For investigators who want to test hypoxia effects for their own cells and projects, these small chambers could be a good introduction. They hold up to twelve 10-cm dishes and require additional equipment for gassing the chamber, including regulators, tubing, and pumps. They must be recharged after each entry, and currently cannot be monitored for internal conditions. Humidity is maintained by including an extra dish with sterile water.

Most hypoxic culturing is performed in a so-called "tri-gas" incubator, though this is a misnomer. Only two gases are supplied—carbon dioxide (as usual) and nitrogen—to reduce the oxygen levels. Generally oxygen can be lowered to 0.5–1%. While some manufacturers may claim oxygen levels as low as 0.1%, this is not truly possible due to sensor detection limits, plus residual oxygen remaining in culture media and plastic culture vessels.[3] Some small incubators purport to offer hypoxic conditions, but these are not ideal since they use a gas mixture from a single tank, and generally do not have separate sensors; therefore it is impossible to ensure that cells are receiving proper amounts of carbon dioxide, oxygen, and nitrogen gases.

Figure 2 - Segmented inner glass door of a Thermo Scientific Heracell tri-gas incubator.

Tri-gas incubator

The first commercially available tri-gas incubator was offered by **Thermo Fisher Scientific** in 1979, only two years after the first paper was published,[4] proving that cells in hypoxia grew better, healthier, and with longer lifespans. Designing a tri-gas incubator is complicated, since adding in a large amount of nitrogen gas (75–95%) affects all the other conditions, including CO_2; temperature; and, of course, humidity. A divided or segmented inner glass door (see *Figure 2*) can help conserve gases and reduce the chances of contaminants entering the incubator.

For human applications, it is important to ensure that the chosen incubator is certified for use with human patient samples. Consider contamination control technologies, since any contamination in cells for therapies or products for use in humans could be disastrous. It is important to have an automated decontamination system that eliminates handling of internal parts, and it should be proven effective by independent testing. In addition, a contamination prevention feature that is constantly working is also vitally important for these sensitive applications.

Conclusion

As cell culturing advances and new technologies mean that cells themselves or cell products will be used for human therapies, culturing conditions become even more important. One critical aspect is to best mimic conditions in the body where the cells originated, including providing hypoxic conditions that

simulate physiological oxygen concentrations. "Tri-gas" incubators efficiently provide these conditions, but similar technologies do not necessarily produce the same results; thus it is important to carefully evaluate manufacturer data when choosing an incubator that provides variable oxygen culturing.

References

1. Panchision, D.M. The role of oxygen in regulating neural stem cells in development and disease. *J. Cell. Physiol*. Sept **2009**, *220*(3), 562–8.
2. Ivanovic, Z. Hypoxia or in situ normoxia: the stem cell paradigm. *J. Cell. Physiol*. May **2009**, *219*(2), 271–5.
3. Richter, A.; Sanford, K. et al. Influence of oxygen and culture media on plating efficiency of some mammalian tissue cells. *J. Nat. Cancer Inst.* **1972**, *49*, 1705–12.
4. Packer, L.; Fuehr, K. Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature* **1977**, *267*, 423–5.
5. Parrinello, S.; Samper, E. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nature Cell Biol.* Aug **2003**, *5*(8), 741–7.
6. Atkuri, K.R.; Herzenberg, L.A. et al. Importance of culturing primary lymphocytes at physiological oxygen levels. *Proc. Nat. Acad. Sci.* **2007**, *104*(11), 4547–52.
7. Wernerspach, D.; Morris, J. Oxygen: too much of a good thing. *Lab. Equip.* **2009**.
8. Lengner, C.J.; Gimelbrant, A.A. et al. Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* **2010**, *141*(5), 872–83.
9. Cooper, P.; Burt, A. et al. Critical effect of oxygen tension on rate of growth of animal cells in continuous suspended culture. *Nature* **1958**, *182*, 1508–9.
10. Danet, G.H.; Pan, Y. et al. Expansion of human SCID-repopulating cells under hypoxic conditions. *J. Clin. Invest.* **2003**, *112*, 126–5.
11. Simiantonaki, N.; Jayasinghe, C. et al. Hypoxia-induced epithelial VEGF-C/VEGFR-3 upregulation in carcinoma cell lines. *Int. J. Oncol.* **2008**, *32*, 585–92.
12. Brahimi-Horn, M.C.; Chiche, J. et al. Hypoxia and cancer. *J. Molec. Med.* **2007**, *85*(12), 1301–7.

Mary Kay Bates, M.S., is Global Cell Culture Specialist, Thermo Fisher Scientific, 275 Aiken Rd., Asheville, NC 28804, U.S.A.; tel.: 218-525-2293; fax: 218-525-1156; e-mail: marykay.bates@ thermofisher.com.