

Benefits of Oxygen Control in the Cell Culture Incubator



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This White Paper outlines the importance of proper homeostasis maintenance in *in vivo* cell culture and oxygen levels *in vivo*.

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Mammalian Cell Culture and the Future of Medicine and Disease Control

Mammalian cell culture is an extremely valuable tool in the direct study of cells and tissue. Emerging regenerative medicine allows cell culture to be used as a direct therapeutic process for patients and others and serve as model systems *in vitro*.

As cell culture techniques are refined, the need to simulate accurate *in vivo* conditions within an *in vitro* environment has become very important. This is especially true in applications involving stem cells, biotherapeutics and embryos.

Environmental deviations from the *in vivo* surroundings from which a particular cell is derived may lead to erroneous data, non-reproducible experiments and unwanted extensions in experimental and drug development processes.



Physiological Oxygen: Understanding Oxygen Levels *in vivo*

Normoxia is the term most often used to describe atmospheric levels of oxygen. This range generally resides between 20-21% O₂ (160 mmHg), though most tissues do not experience oxygen levels at 20-21%.¹

- In our lungs, oxygen levels are around 14.5% or as low as 3.4-6.8% in peripheral tissues. Physiological oxygen (2-10%) is therefore considered 'hypoxic' in respect to atmospheric oxygen.
- Most organs in the body, including the brain, liver and pancreas, reside between 2-10% O₂ while certain tissues, such as the thymus and areas of the kidney, reside at oxygen concentrations lower than 1%.²
- Pathological tissue like cancer, contains an oxygen gradient in which inter-cellular oxygen levels can reach below 1%.
- Hypoxic oxygen levels regulate a variety of important and normal pathological and physiological processes, such as cell differentiation, proliferation, wound healing and fetal development. Additionally, oxygen levels dictate essential events in early embryo development and stem cell niches.³

Table 1 – Relative Oxygen from Ambient to Below Ambient

Oxygen	Description
21.0%	Oxygen in air at normal atmospheric pressure
19.9%	Oxygen in cell culture incubator operating at 5% CO ₂
13.5%	Inspired oxygen pO ₂ in alveoli. O ₂ is affected by inflow and outflow of gases and water vapor
9.5%	Arterial blood oxygen concentration
6.5%	Approximate pO ₂ at venous end or circulation

Importance of Homeostasis in Cell Culture

Maintenance of a successful *in vivo* environment within cell culture lies in the principal of properly maintaining cellular homeostasis. Cells require a delicate balance of temperature, pH, nutrients and ions through an intricate network of cellular processes.⁴

In the early days of cell culture, scientists were able to successfully grow mammalian cells *in vitro* by exposing them to certain environmental elements that contribute to homeostasis. Today, most mammalian cells are maintained at CO₂ levels between 5-7% and supplied with a strict formulation of nutrients, pH buffers and growth factors.

These components have led to significant improvements in cell culture technology and healthier cell proliferation. Yet oxygen levels that are optimized for growth of specific cells are often overlooked.

Table 2 – Known Percentages of Oxygen in Selected Human Tumors⁵

Tumor Type	Median Oxygen, Normal Tissue	Media Oxygen, Tumor
Brain	3.4%	1.7%
Head and Neck	5.3% to 6.7%	1.6% to 1.9%
Lung	5.6%	1.9% to 2.2%
Breast	6.8%	1.3%
Cervix	5.5%	1.2%
Liver	3.9%	1.8%
Pancreas		0.3%
Prostate	3.4% to 3.9%	0.3% to 1.2%
Vulva		1.3% to 1.7%
Melanoma	5.3%	1.5%
Renal	4.9%	1.3%
Rectal	6.8%	2.5% to 4.2%
Sarcoma	6.7%	1.8%

Atmospheric air is comprised of 21% oxygen, 78% nitrogen and 1% trace gases. Once breathed into the lungs, oxygen molecules are transported by the hemoglobin molecules on red blood cells through the bloodstream and carried to all organs in the body.

When a cell culture incubator is configured to create a typical 5% CO₂ atmosphere, O₂ levels are reduced to 19.95%. Although this O₂ level is somewhat reduced, hypoxia is not achieved nor is it controlled to the point of reproducibility and continuity.

At the cellular level, *in vitro* oxygen plays a critical role in the regulation of a variety of cellular processes.⁶ Within a given tissue, the oxygen level controls specific biological events, such as cell differentiation, cell proliferation and wound healing.

Conventionally, mammalian cells have been cultured at atmospheric oxygen levels (~20%) *in vitro*. Most mammalian cells, however, are derived from tissues that contain sub-atmospheric oxygen in the range of 2-11%.⁷

While *in vitro* cell culture remains a mainstay in the modeling of disease and physiological mechanisms, contemporary research demonstrates the detrimental effects of culturing mammalian cells at atmospheric oxygen levels.

Research has linked higher *in vitro* oxygen levels to more frequent chromosomal breaking, expression of 'stress response' genes, more oxidative damage and a weaker cell overall. Whether cultures are used for hosting or for expression of a cellular derived product, incompatible O₂ exposure impacts downstream results.

Oxygen Regulation of Hypoxic Inducible Factors in the Cell Culture Processes

Below ambient oxygen concentrations function as a signaling molecule for certain cellular events. Among the most significant cellular factor regulated by low oxygen concentrations is hypoxia-inducible factor (HIF).

HIF embraces a class of DNA-binding proteins and becomes activated at physiological oxygen levels. HIF regulates the transcription of a variety of genes critical for maintaining overall cellular homeostasis and key pathways, such as angiogenesis, glycolysis and erythropoiesis.

Genes regulated by HIF are important for improving cell survival and reducing cell stress which occurs physiologically during fetal development or ischemia.⁸

HIF also promotes normal physiological cell differentiation and proliferation by regulating the expression of genes that control cellular glucose uptake and metabolism. One gene, vascular endothelial growth factor (VEGF-A), is critical for the cellular process of angiogenesis (blood vessel growth) and is upregulated with the presence of HIF.

Blood vessel growth helps facilitate oxygen delivery to hypoxic tissues. VEGF also has a key role in fetal development where hypoxic microenvironments are typical.⁹

In addition, HIF also has an important role in pathophysiologic or disease conditions, such as cancer, where abnormalities often create hypoxic states in certain tissues. Rapid cell division during tumor progression generates hypoxic conditions that lead to activation of HIF, which, in turn, initiates cell survival processes that maintain homeostasis and cancer growth (metastasis).¹⁰

Because physiological oxygen concentrations are critical for regulating a multitude of cell culture processes, atmospheric oxygen levels can create unintended side effects, such as reactive oxygen species (ROS) at higher oxygen concentrations.

ROS affect cellular processes by damaging DNA, modifying protein synthesis and function and causing cell membrane instability.¹¹ Cell cultures grown in atmospheric oxygen express a stress response gene signature which is different than cells cultured in physiological oxygen.

Consequently, using these cells for downstream cell culture experiments may have unpredictable results. Culturing cells at physiological oxygen levels brings cellular metabolism and homeostasis to a much closer *in vivo* level and activates key factors, such as HIF, which has been shown to promote cell adaptation and survival.

HIF in turn has a multitude of effects that allow cells to grow faster, live longer, stay healthier and have a more normal gene expression profile.¹²

In atmospheric oxygen, HIF is produced in low or undetectable numbers. Here, excess oxygen leads to detrimental cellular consequences, such as oxidative damage and upregulation of stress proteins.

In Vitro Fertilization

With an increasing amount of research citing the benefits of culturing cells at non-atmospheric oxygen levels, it is likely that physiological oxygen (~5%) and hypoxic (~0.1%~2.5%) studies will increase in the future.

Researchers in the field of *in vitro* fertilization (IVF) have already realized the benefits of physiological oxygen and pioneered its use in their cultures.

Physiological oxygen is a stimulus for the expression of certain factors that play a role in embryogenesis. With the strict conditional nature of embryo development and host implantation for fertilization, IVF researchers demand an optimized *in vitro* environment for embryo cultivation.

Numerous studies have demonstrated that embryos cultured at elevated O₂ concentration levels are impacted negatively during their development, whereas culturing with low oxygen (5%) levels results in improved embryo quality.

Studies have also shown that 5% O₂ (or physiological oxygen) yields higher quality embryos, implantation and better pregnancy success than 20% O₂ (atmospheric oxygen) culturing.¹³

HIFs in Embryology

HIF is one of several factors important for the success of embryo development. During embryogenesis and organogenesis, there are many hypoxic microenvironments that form; HIF is expressed to adapt to these situations.

In one study, HIF-deficient embryos, a similar phenotype as those that might be cultured at atmospheric oxygen levels, had defects in blood vessel formation and neural-fold closure.¹⁴ These results support the role that HIF plays in angiogenesis and cell proliferation.

Another study demonstrated the role of HIF in the proper formation of a placental architecture and its subsequent vascularization. Other studies have characterized the increased expression of HIF proteins during placental development.

Taking these studies into account suggests that the hypoxic environment that embryos encounter normally *in vivo* is a critical factor for at least their cardiovascular-pulmonary development *in vitro*.

IVF researchers must reproduce an accurate *in vivo* environment for embryos. Reactive oxygen species (ROS) is one variable that is diminished when culturing embryos at physiological oxygen levels.

Researchers have recognized that the deleterious effects of ROS on DNA, proteins and other biological molecules can significantly affect embryo development and eventual implantation or fertilization.¹⁵

Thus, growing embryos at physiological oxygen levels is beneficial for not only replicating appropriate *in vivo* cellular homeostasis, but also ensuring better integrity of the embryo's genetic profile. All of these benefits taken together can make a large difference in experimental results as well as implantation and fertilization success.

Physiological Oxygen in Other Applications

The success of culturing embryos in IVF suggests that altering the oxygen levels in other applications will similarly improve cell health and growth.

The environmental principles that dictate cell differentiation and proliferation can be easily translatable to other research areas, such as stem cell research or cancer biology.

Oxygen is a factor in several metabolic and cellular pathways. Thus, to be truly comprehensive and to obtain the most relevant results, researchers studying mammalian cells should consider culturing their cells in physiologically relevant oxygen conditions.

Two major areas that have made significant headway in low oxygen studies are stem cell and cancer research.

Table 3 – Contextual Oxygen and Terminology¹⁶

Oxygen	Pressure	Percentage	Scientific Term	Common Term
In Air	160 mm/Hg	21%	Atmospheric	Normoxic
pO ₂ <i>in vivo</i>				Hypoxic
Lung	150 mm/Hg	2% to 10%	Patho-physiological	
Circulation	50 to 100 mm/Hg			
Tissue	20 to 50 mm/Hg			
Hypoxia	<15 mm/Hg	<2%	Physiologic	

Stem Cells

An understanding of physiological oxygen has become increasingly critical in stem cell research. Research has shown that long-term culture of stem cells at atmospheric oxygen give rise to chromosomal abnormalities as well as genomic and epigenomic aberrations.¹⁷

These mutations could lead to tumorigenesis or affected downstream results. Because many types of stem and progenitor cells often reside in a physiological microenvironment with oxygen between 1-5%, exposure to hyperoxic conditions may lead to deleterious effects.

When investigators seeking to corroborate this hypothesis cultured stem cells under physiological oxygen concentrations, the outcome was better genomic stability and maintenance of stemness in adult stem and embryonic stem (ES) cells.¹⁸

Physiological oxygen levels have also been shown to influence stem cell growth rates and differentiation. One study found that culturing rat bone marrow mesenchymal stem cells in 5% oxygen yielded increased proliferation and colony-forming ability than when cultured at 20% oxygen (atmospheric).¹⁹

Furthermore, ES cells revealed more robust growth under hypoxic oxygen conditions with a significant reduction in differentiation as compared to ES cells exposed to ambient air. Hypoxic conditions helped promote full pluripotency of embryonic stem cells.

In contrast, culturing rat peripheral and central nervous system stem cells at physiological oxygen levels promoted their differentiation into neurons. It appears that oxygen's modulation of stem cell fate is a complex mechanism dependent on the tissue. This data is an example of continuously evolving research that represents the role oxygen control plays in stem cell research.

While oxygen regulates stem cell differentiation and genomic integrity in ways still not completely understood, researchers have been studying the effects of oxygen via HIF. Results suggest that HIFs affect stem and progenitor cell differentiation. In one study, it was shown that trophoblast stem cell differentiation in placenta development was altered in HIF-deficiency in mice.²⁰

The lack of HIF, which represents a phenotype similar to atmospheric oxygen, resulted in stem cells differentiating into another type of cell, e.g. trophoblast giant cells instead of spongiotrophoblasts. This study suggested that physiological oxygen levels (3% in this tissue) activated HIF, which in turn pushed placental cells to differentiate into a specific fate.

Cancer and Physiological Oxygen

Oxygen levels play a key role in pathophysiological studies. Viruses, cancer and other disease states often create hypoxic microenvironments in tissues where HIF initiates complex interactions.

In a study seeking to understand the mechanism of neurotoxicity during HIV infection, cells cultured at physiological oxygen experienced cell death from HIV's neurotoxins, while those cultured at atmospheric oxygen did not.²¹ This implies that the mechanism through which HIV killed cells relied heavily on the appropriate levels of oxygen.

In another study, HIF via hypoxia activation was found to downregulate B-catenin in the Wnt signaling pathway by competitively binding to B-catenin resulting in cell-cycle arrest and inhibition of transcriptional activity.²²

B-catenin and Wnt signaling have critical roles in embryogenesis, but have also been implicated in cancer development and progression when improperly regulated. These studies suggest that oxygen and HIF levels have a role in many pathophysiological processes.

As more research is conducted to understand the mechanisms of cancer and other diseases, it is critical to examine how these cells are affected by oxygen concentrations.

To achieve the most relevant results, lessons learned from IVF can be applied to other areas, such as toxicology and cancer biology. Better understanding how oxygen factors into any biological mechanism is of supreme importance for further research development.

Conclusion

With current trends moving towards incorporating oxygen into investigations, particularly in applications such as *in vitro* fertilization, stem cell and cancer biology, more investigators are examining options associated with conventional CO₂ incubators that extend to O₂ control as well.

Due to budget restrictions, the need for a cell culture incubator with convertible performance, from conventional CO₂ control to CO₂ and O₂ control is critical.

Advances in incubator design now include the addition of multiple gas control systems based on a new generation of dual-infrared CO₂ sensors, zirconia O₂ sensors and algorithms configured to assure quick recovery of desired atmosphere levels following door openings.²³

Coupled with both active and passive contamination mitigation, along with a range of decontamination options to meet FDA criteria, the focus of contemporary cell culture research continues to narrow on cell specificity.

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