

REVIEW ARTICLE

Technical aspects of oxygen level regulation in primary cell cultures: A review

Mazyar YAZDANI

Department of Biosciences, University of Oslo, Oslo, Norway

ITX093416A02 • Received: 23 June 2016 • Revised: 19 December 2016 • Accepted: 20 December 2016

ABSTRACT

Oxygen (O₂) is an essential element for aerobic respiration. Atmospheric concentration of O₂ is approximately 21%. Mammalian cells, however, are generally adapted to O₂ levels much lower than atmospheric conditions. The pericellular levels of O₂ must also be maintained within a fairly narrow range to meet the demands of cells. This applies equally to cells *in vivo* and cells in primary cultures. There has been growing interest in the performance of cell culture experiments under various O₂ levels to study molecular and cellular responses. To this end, a range of technologies (e.g. gas-permeable technology) and instruments (e.g. gas-tight boxes and gas-controlled incubators) have been developed. It should be noted, however, that some of these have limitations and they are still undergoing refinement. Nevertheless, better results should be possible when technical concerns are taken into account. This paper aims to review various aspects of O₂ level adjustment in primary cell cultures, regulation of pericellular O₂ gradients and possible effects of the cell culture medium.

KEY WORDS: O₂ level adjustment; pericellular O₂ gradients regulation; culture medium effects; primary cell cultures

Introduction

The appearance of multicellular life during the Earth's history has been linked to oxygen (O₂) levels in the environment. Increased O₂ levels enabled the shift from the inefficient anaerobic respiration found in prokaryotes to more efficient aerobic respiration in eukaryotes, providing the eukaryotic cells with access to more energy for the energy-demanding cellular processes required for a multicellular existence (Hedges *et al.*, 2004). An appropriate supply of O₂ to tissues is necessary for their optimal function and continued survival.

Today O₂ makes up approximately 21% of the Earth's atmosphere, but mammalian cells are generally adapted to lower concentrations. The concentration decrease occurs during the inhalation and transportation processes. The delivery of O₂ is determined by the metabolic requirements and functional status of each organ and tissue. The balance between delivery and consumption determines the O₂ partial pressure (pO₂), which is specific to each organ and generally much lower than that of the atmosphere (Carreau *et al.*, 2011).

Since the mid 1980s, ethical and economical issues along with other factors have encouraged the development of cell culture techniques (*in vitro*) instead of using traditional whole-animal experiments (*in vivo*) in biological and medical studies (Hayes 2014). Since the *in vitro* environment is fundamentally different from the *in vivo* physiological environment, much effort has gone into adapting conditions for *in vitro* cell culture to be more like *in vivo* conditions (e.g. Grzelak *et al.*, 2001; Ruch *et al.*, 1989; Yazdani *et al.*, 2015). Among the several factors important for cellular processes, O₂ is one of the most important. It plays a crucial role in many cellular processes ranging from metabolism to signalling. Ideally then, its level should be precisely controlled. However, the convenience of working with cell cultures in the ambient atmosphere, historical precedence and the absence of suitable methods and appropriate instruments for precise regulation of pericellular O₂ gradients during experiments have led to cells being exposed to higher O₂ levels than they would normally experience *in vivo*. This, predictably, results in changes within the cells such as altered phenotypes and gene-expression levels (Satoru & Kiyoshi 2012; Wion *et al.*, 2011). Despite these alterations to cellular functions and the increased formation of reactive O₂ species, it is sometimes desirable to use primary culture models under high levels of O₂. For example, an oxygenated co-culture of hepatocytes and endothelial cells has previously been shown to be a useful tool to predict *in*

Correspondence address:

Dr. Mazyar Yazdani

Department of Biosciences, University of Oslo
P.O.Box 1066 Blindern, N-0316 Oslo, Norway
TEL.: +47 22857314 • FAX +47 22854438
E-MAIL: mazyaryazdani.edu@gmail.com

vivo drug clearance (Kidambi *et al.*, 2009). However, for *in vitro* studies attempting to emulate *in vivo* processes it is vital that the pericellular environments are as comparable as possible to the natural state.

With the importance of O₂ in molecular and cellular responses, there has been growing interest in the performance of cell culture experiments under various O₂ levels. This has required novel *in vitro* culture systems to overcome the difficulties associated with the delivery of precise O₂ levels. Advancement in the design of cultureware has led to improved gas exchange between cells and the surrounding microenvironment. For example, the use of gas-permeable technology for cultivating adherent (hydrophilic surface) and suspension (hydrophobic surface) cells provided better ventilation of cultures. Additionally, several apparatuses have been developed to maintain desired atmospheric conditions. These include the conventional culture apparatuses such as gas-tight boxes and gas-controlled incubators as well as more advanced equipment that can provide stringent control of O₂ throughout the culture period (Gille & Joenje 1992; Satoru & Kiyoshi 2012). It should be noted, however, that some of these technologies and instruments have limitations and are still being refined. Nevertheless, better results should be possible when technical concerns are taken into account. This paper reviews some aspects of O₂ level adjustment in primary cell cultures, regulation of pericellular O₂ gradients, and possible effects of the cell culture medium.

Adjustment of O₂ levels *in vitro*

The Earth's atmosphere is composed of a mix of several gases: approximately 78% nitrogen (N₂) and 21% O₂, with traces of argon (Ar, 0.9%), carbon dioxide (CO₂, 0.03%), water vapor, and various other components. Mammals require oxygen (O₂) in order to generate ATP during aerobic metabolism. The level of O₂ decreases from atmospheric levels following inhalation by the respiratory system (19.7% – 14.5%; 150 – 110 mmHg), transportation through arterial blood (13.2%; 100 mmHg) and delivery to the body tissues (7% – 0.7%; 50 – 5 mmHg). The levels of O₂ must be maintained within a fairly narrow range at each stage to respond to cellular demands. Hence, any changes in the physiological environment of the body that influences the level of O₂ (*e.g.* pathological conditions) can result in changes in the cells. Similarly may stress be induced when the cells are isolated from their organ and kept under culture conditions at O₂ levels different from physioxia (*i.e.* the *in vivo* condition). Levels of O₂ higher and lower than physioxia are defined as hyperoxia and hypoxia, respectively. Thus 21% of O₂ is considered hyperoxic for freshly isolated cells (Carreau *et al.*, 2011; Wion *et al.*, 2011).

Several apparatuses have been developed to control the O₂ levels of cell cultures. They may be used to maintain culture conditions close to physioxia status or to study the cellular effects of hypoxia and hyperoxia. Glass culture flasks equipped with silicone stoppers are among

Table 1. An overview of the commercially available gas-tight/flush boxes with associated product information.

	Commercial Name	Model	Size ¹	Supplier
	Hypoxia/modular incubator chamber, Flush box	MIC	12"D x 4.7"H	STEMCELL Technologies, Inc., Billups-Rothenberg Inc., BioSpherix, Ltd.
	Hypoxia chamber for cell culture	C-Chamber		BioSpherix, Ltd.
		1-shelf chamber	14"W x 12"D x 5.25"H	
		2-shelf chamber	14"W x 12"D x 6"H	
		3-shelf chamber	14"W x 12"D x 8"H	
	4-shelf chamber	14"W x 12"D x 10"H		
	O ₂ control cabinet for <i>in vitro</i> studies	Model 1 (1-shelf chamber)	16"W x 15"D x 9.5"H	Coy Laboratory Products, Inc.
		Model 2 (2-shelf chamber)	16"W x 15"D x 11.5"H	
		Model 3 (3-shelf chamber)	16"W x 15"D x 14"H	
		Model 4 (4-shelf chamber)	16"W x 15"D x 16.25"H	

¹ External dimensions.

the simplest instruments for this purpose. The flask, after receiving the cells, is flushed with the desired gas mixture and then tightly sealed with a silicone stopper (and placed in an incubator if a particular temperature is desired). Further examples of a closed culture system are gas-tight or flush boxes (Table 1). These classic re-sealable chambers are designed to hold cell culture plates inside. Seal integrity is a vital aspect of this system as debris blockage, human error and degraded seal gaskets may cause air leakage. Equipping the boxes with feedback control devices (Table 2), rather than using gas leak sound as an indicator, would enable much more accurate and reliable control and monitoring of the internal gases. The gas-tight boxes are very practicable, available in a range of sizes, portable and they can be placed inside an incubator while maintaining constant O₂ levels inside the chamber. Gas-controlled incubators are also available (e.g. tri-gas incubators) for controlling the O₂ levels of cell cultures. These however suffer from the drawbacks of being less portable than simple gas-tight boxes and are also more susceptible to fluctuations in internal gas composition due to the repeated opening and closing of the device doors (Gille & Joenje 1992). Efforts continue in the development of separate gas-tight boxes and incubator systems, as well as combined forms (e.g. Satoru & Kiyoshi, 2012).

To attain the desired experimental concentrations, O₂ (solute gas) must be mixed with other gases (balance gases). Nitrogen (N₂) is commonly used alone for this purpose, but some researchers use also argon (Ar). These two gases (N₂ and Ar) are inert (i.e. do not undergo chemical reactions under experimental conditions) and their price is relatively low. Another common solute

gas for cell culture is carbon dioxide (CO₂). It interacts with the bicarbonate buffer in the cell culture medium, stabilizing the pH at about the optimum level (~7.4). CO₂ may be excluded from the gas mixture if the culture is supplemented with a CO₂-independent buffering system, such as HEPES (Andersen & Jørgensen 1995; Minuth *et al.*, 2010; Williamson & Cox 1968).

Gases are available in two forms: as premixed or pure gas tanks. Premixed gases are convenient for many purposes but the tanks are often device specific (e.g. tri-gas incubator). Pure gases allow researchers to tailor gas mixtures to their own requirements, but they require on-site blending before use. To avoid nonhomogeneous gas concentration in the latter case, a mixing station may be used to pre-mix gases before they are introduced into the working system (Satoru & Kiyoshi 2012). The blending can be performed manually or, preferably, using instruments designed for dynamic gas-mixture preparation. The latter allow for complete control over final gas composition. In any case, certified medical grade gases should be used.

O₂ gradient in the microenvironment of the cell

In order to be available for cells in culture, O₂ molecules must be transferred from the gas phase (atmospheric environment) to the liquid phase (culture medium). The rate and extent to which O₂ equilibrium is reached in a medium is a function of the surrounding O₂ level, the oxygenation method, culture temperature and the volume and ionic strength of the medium (Gstraunthaler *et al.*, 1999). Hence, cell handling and culturing in conventional

Table 2. Specifications and product information for gas-tight/flush box gas controllers.

Model	Function	Control range	Accuracy	Resolution (precision)	Supplier
ProOx P110	Control O ₂ in any semi-sealable chamber	0.1–99.9% O ₂	±1% at constant temperature/pressure ±2% full scale over operating temperature range	0.1%	BioSpherix, Ltd.
ProOx C21	Control O ₂ and CO ₂ in any chamber	0.1–99.9% O ₂ , 0.1–20% CO ₂	O ₂ : ±1% at constant temperature/pressure ±2% over entire temperature range. CO ₂ : .1% or 5% of measurement, whichever is greater	0.1%	BioSpherix, Ltd.
ProCO2 P120	Control CO ₂ in any chamber	0.1–20% CO ₂	±.3% (@0%) to ±.7% (@20%) at 25°C and 1013hPa	0.1%	BioSpherix, Ltd.
OxyCycler C42	Control O ₂ and CO ₂ in multiple chambers	O ₂ : 0.1–99.9%, CO ₂ : 0.1–20%	O ₂ : ±1% at constant temperature and pressure ±2% at entire temperature range. CO ₂ : ±5% or 0.1%, whichever is greater	0.1%	BioSpherix, Ltd.
O ₂ controller	Control O ₂ and N ₂ /air (or a mixture of CO ₂ and N ₂ /air)	0–100% or 0–60% O ₂ atmospheric	– ³	0.1%	Coy Laboratory Products, Inc.
CO ₂ controller	Control CO ₂ and N ₂ /air	0–19.9% in 0.1% increments	<+/- [0.02% CO ₂ +2% of reading]	0.1%	Coy Laboratory Products, Inc.
Single flow meter	Control the gas flow of premixed gases	Calibrated for 0.1–10 LPM ¹	–	–	STEMCELL Technologies, Inc. Billups-Rothenberg Inc.
Dual flow meter ²	Used to mix gases in addition to controlling the gas flow rate	Calibrated for 0.1–1.0 and 0.1–10 LPM	–	–	STEMCELL Technologies, Inc. Billups-Rothenberg Inc.

¹ LPM: Litres per minute

² The dual flow meter should be used in combination with an O₂ detector.

³ Blank fields represent information lacking from the supplier.

clean benches, under ambient atmosphere, means that excess O₂ is dissolved in the medium. There is thus a lag period after inoculation of the cultures prior to the attainment of O₂ equilibrium in the experimental apparatus. A better practice would be to use a medium that was previously kept at the desired O₂ level prior to culturing (Wion *et al.*, 2011). In addition, the O₂ remaining inside the culture-ware, after culture preparation, requires several hours to reach equilibrium with the adjusted O₂ level using apertures (Satoru & Kiyoshi 2012; Westfall *et al.*, 2008). Best practice would be to handle cultures under a covered bench with an atmosphere controlled to the desired endpoint condition.

Due to normal metabolic processes, pericellular O₂ gradients form in primary cell cultures. As a result, a continuous replenishment of oxygen is required to avoid hypoxic conditions adjacent to the culture. This is of particular concern in high cell-density cultures. The implementation of lab techniques, including careful shaking of the cell culture at regular intervals, employment of gas-permeable technology such as polystyrene film on culture-ware, and modifying product designs such as flasks having a filter screw cap or holes with filters may facilitate the process, but they do not go far enough. The inclusion of tracheal spaces (connected to an external gas supply) immediately beneath the gas-permeable layer that cells are already cultured on would provide better gas exchange between the cells and the desired atmosphere. It also eliminates the need for a gas-liquid interface. This continuous flow of O₂ may be advantageous over traditional monolayer culture systems, in which gas exchange occurs only through the medium. However, it still lacks precise regulation of the O₂ microgradient during the experiment. The introduction of microsystem techniques to cell culture applications is a fast-growing field and one that offers precise sensing and patterning of microgradients. In an example, Park *et al.*, (2006) designed a device for controlling and changing the spatial and temporal profile of the O₂ microgradient in monolayer cultures using microscale electrolysis visualized by fluorophore-impregnated films.

An alternative to the static cell culture systems mentioned so far are perfusion culture systems, which provide continuous nutrition and respiratory gas through a constant flow or in pulses of medium. In these systems, O₂ can be supplied to the culture by sparging, membrane diffusion and medium perfusion. In the latter method, the medium may be perfused through an oxygenation chamber before it enters the culture system, ensuring constant supplementation of O₂. One example is the modular culture system developed by Minuth *et al.*, (2010) for the generation of multiple specialized tissues. Their system is equipped with a spiral of long, thin-walled, highly gas-permeable silicon tube for optimal diffusion of O₂ when the medium passes through.

Given the many oxygenation methods used by different researchers, how these methods affect the O₂ levels experienced by cells in culture, and the consequent ramifications on the results of studies, it is important

that they be reported accurately. To this end, Wion *et al.*, (2011) called for improved reporting of the utilized oxygenation methods and occurrence of O₂ gradients in cellular microenvironments. One important factor to include in such a description would be how the reported O₂ levels were selected, i.e. was it based on a set value of instrument (*e.g.* incubator), the bulk medium, or on the pericellular environment (Wion *et al.*, 2011).

Culture medium effects

In addition to allowing gas exchange between the cells and the surrounding atmosphere, the culture medium also provides nutrients and energy required for cell growth and maintains pH and osmolality. Too little attention has been paid to the effects of the medium's composition on the O₂ level. For example, Nahmias *et al.*, (2006) showed that fetal bovine serum plays a role in the cellular reaction to O₂ levels. The authors identified a negative effect of serum on O₂-enhanced metabolism of primary rat hepatocytes cultured on an O₂-carrying matrix.

In addition to the composition, the volume of the medium (particularly its depth over monolayer cultures) is also of concern. In static systems where oxygenation only occurs through surface aeration, the O₂ transfer rate (OTR) depends on a liquid surface area adequate to the volume ratio of the medium. The optimal level of the medium is suggested to be 0.2 cm, equivalent to a volume of 0.2 mL/cm². Greater volumes can result in decreased OTR (Gstraunthaler *et al.*, 1999; McAteer & Davis 1994). In this regard, monolayer cultures of renal tubular epithelia were shown to be affected when the medium volume covering them was increased. This gave rise to a decrease in the supply of O₂ resulting in a shift from oxidative metabolism to increased rate of glycolysis (Gstraunthaler *et al.*, 1999). Since the medium is an important part of cell culture, further studies are needed to better understand its possible effects on O₂ level in the pericellular environment.

Applications of regulated O₂ levels *in vitro*

The apparatuses mentioned above may be used to maintain physioxia status in cell culture or to study the cellular effects of hypoxia and hyperoxia. Such capabilities would allow further research into, for example, hyperbaric oxygen treatment (HBO) topics including wound healing (Malda *et al.*, 2007), cancer treatment (Moen & Stuhr 2012), neurogenesis (Mu *et al.*, 2011), etc. Furthermore, such investigations would facilitate the development of more effective HBO therapies.

In addition to basic research, many advanced applications of cell culture can benefit from controlled O₂ concentrations. For example, the culture of human embryos for *in vitro* fertilization under conditions close to physioxia status eliminates transmission of hyperoxia-associated abnormalities (*e.g.* genotoxicity) to the offspring (Satoru &

Kiyoshi 2012). Another benefit would concern cell-based therapies, such as stem cell treatments, as most stem cells experience hypoxia *in vivo* (Bates 2012; Wion *et al.*, 2011). The clinical application of cell culture derived products for personalized medicines, such as monoclonal antibodies for cancer treatments, also requires precise regulation of O₂ levels during production (Bates 2012). In order to assess the safety of drugs, chemicals, cosmetics, and consumer products, a variety of cell-based tests and tissue models have been developed. The results of risk assessments may be adversely affected by non-physiological conditions, including uncontrolled O₂ concentrations, with resultant consequences for human health (Bates 2012).

Conclusion

Cells *in vivo* are exposed to O₂ levels much lower than atmospheric levels due to the gradual decrease of environmental O₂ levels resulting from transportation into the body and to the tissues. Thus handling and culturing of freshly isolated cells in conventional clean benches under ambient atmosphere causes hyperoxia. With increasing interest in the performance of cell culture experiments under various O₂ levels, a range of technologies (*e.g.* gas-permeable technology) and instruments (*e.g.* gas-tight boxes and gas-controlled incubators) has been developed. However, some of these have limitations and require further development. Nevertheless, better results should be possible if technical concerns were taken into account. In this paper some such aspects of O₂ level adjustment in primary cell cultures, regulation of pericellular O₂ gradients and possible effects of cell culture medium have been reviewed.

Competing interests statement

I declare the author has no competing interests as defined by De Gruyter, or other interests that might be perceived to influence the interpretation of the article.

REFERENCES

- Andersen CY, Jørgensen N (1995). Improvement of sperm motility by the addition of progesterone to the Percoll medium during sperm purification. *Hum Reprod* **10**: 3183–3185.
- Bates MK (2012). Culturing cells under hypoxic conditions for biologically relevant results. *Am Lab*.
- Carreau A, Hafny-Rahbi BE, Matejuk A, Grillon C, Kieda C (2011). Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med* **15**: 1239–1253.
- Gille JJP, Joenje H (1992). Cell culture models for oxidative stress: Superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mutat Res* **275**: 405–414.
- Grzelak A, Rychlik B, Bartosz G (2001). Light-dependent generation of reactive oxygen species in cell culture media. *Free Radic Biol Med* **30**: 1418–1425.
- Gstraunthaler G, Seppi T, Pfaller W (1999). Impact of culture conditions, culture media volumes, and glucose content on metabolic properties of renal epithelial cell cultures. *Cell Physiol Biochem* **9**: 150–172.
- Hayes AW (2014) Hayes' principles and methods of toxicology Taylor & Francis Group, LLC, Boca Raton, FL.
- Hedges S, Blair J, Venturi M, Shoe J (2004). A molecular timescale of eukaryote evolution and the rise of complex multicellular life. *BMC Evol Biol* **4**: 2.
- Kidambi S, Yarmush RS, Novik E, Chao P, Yarmush ML, Nahmias Y (2009). Oxygen-mediated enhancement of primary hepatocyte metabolism, functional polarization, gene expression, and drug clearance. *Proceedings of the National Academy of Sciences* **106**: 15714–15719.
- Malda J, Dawson R, Kairuz E, Topping G, Long R, Upton Z (2007). In vitro models for evaluation of hyperbaric oxygen therapy in wound healing: a review. *Diving Hyperb Med* **37**: 25–30.
- McAteer JA, Davis J (1994) Basic cell culture techniques and the maintenance of cell lines. In: Davis JM (ed) Basic Cell Culture: A Practical Approach. IRL Press, Oxford,
- Minuth WW, Denk L, Glashauser A (2010). A modular culture system for the generation of multiple specialized tissues. *Biomaterials* **31**: 2945–2954.
- Moen I, Stuhr LEB (2012). Hyperbaric oxygen therapy and cancer—a review. *Target Oncol* **7**: 233–242.
- Mu J, Krafft PR, Zhang JH (2011). Hyperbaric oxygen therapy promotes neurogenesis: where do we stand? *Med Gas Res* **1**: 1–7.
- Nahmias Y, Kramvis Y, Barbe L, Casali M, Berthiaume F, Yarmush ML (2006). A novel formulation of oxygen-carrying matrix enhances liver-specific function of cultured hepatocytes. *The FASEB Journal* **20**: 2531–2533.
- Park J, Bansal T, Pinelis M, Maharbiz MM (2006). A microsystem for sensing and patterning oxidative microgradients during cell culture. *Lab on a Chip* **6**: 611–622.
- Ruch RJ, Crist KA, Klaunig JE (1989). Effects of culture duration on hydrogen peroxide-induced hepatocyte toxicity. *Toxicol Appl Pharmacol* **100**: 451–464.
- Satoru K, Kiyoshi T (2012) Cell handling and culture under controlled oxygen concentration. In: Biomedical Tissue Culture. InTech. doi: 10.5772/52112
- Westfall SD, Sachdev S, Das P, Hearne LB, Hannink M, Roberts RM, Ezashi T (2008). Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. *Stem Cells Dev* **17**: 869–882.
- Williamson JD, Cox P (1968). Use of a new buffer in the culture of animal cells. *J Gen Virol* **2**: 309–312.
- Wion D, Christen T, Barbier EL, Coles JA (2011). PO₂ matters in stem cell culture. *Cell Stem Cell* **5**: 242–243.
- Yazdani M, Paulsen R, Gjøen T, Hylland K (2015). Reactive oxygen species and cytotoxicity in Rainbow trout hepatocytes: Effects of medium and incubation time. *Bull Environ Contam Toxicol* **94**: 193–198.