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REVIEW ARTICLE

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

Mazyar YAZDANI
Department of Biosciences, University of Oslo, Oslo, Norway

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ABSTRACT
Oxygen (O_2) is an essential element for aerobic respiration. Atmospheric concentration of O_2 is approximately 21%. Mammalian cells, however, are generally adapted to O_2 levels much lower than atmospheric conditions. The pericellular levels of O_2 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 O_2 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 O_2 level adjustment in primary cell cultures, regulation of pericellular O_2 gradients and possible effects of the cell culture medium.

KEY WORDS: O_2 level adjustment; pericellular O_2 gradients regulation; culture medium effects; primary cell cultures

Introduction

The appearance of multicellular life during the Earth's history has been linked to oxygen (O_2) levels in the environment. Increased O_2 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 O_2 to tissues is necessary for their optimal function and continued survival.

Today O_2 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 O_2 is determined by the metabolic requirements and functional status of each organ and tissue. The balance between delivery and consumption determines the O_2 partial pressure (pO_2), 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, O_2 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 O_2 gradients during experiments have led to cells being exposed to higher O_2 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 O_2 species, it is sometimes desirable to use primary culture models under high levels of O_2. 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: mazyar.yazdani.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 O_2 in molecular and cellular responses, there has been growing interest in the performance of cell culture experiments under various O_2 levels. This has required novel *in vitro* culture systems to overcome the difficulties associated with the delivery of precise O_2 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 O_2 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 O_2 level adjustment in primary cell cultures, regulation of pericellular O_2 gradients, and possible effects of the cell culture medium.

Adjustment of O_2 levels *in vitro*

The Earth's atmosphere is composed of a mix of several gases: approximately 78% nitrogen (N_2) and 21% O_2, with traces of argon (Ar, 0.9%), carbon dioxide (CO_2, 0.03%), water vapor, and various other components. Mammals require oxygen (O_2) in order to generate ATP during aerobic metabolism. The level of O_2 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 O_2 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 O_2 (*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 O_2 levels different from physioxia (i.e. the *in vivo* condition). Levels of O_2 higher and lower than physioxia are defined as hyperoxia and hypoxia, respectively. Thus 21% of O_2 is considered hyperoxic for freshly isolated cells (Carreau *et al.*, 2011; Wion *et al.*, 2011).

Several apparatuses have been developed to control the O_2 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 *in vitro* 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.

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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 O_2 levels inside the chamber. Gas-controlled incubators are also available (*e.g.* tri-gas incubators) for controlling the O_2 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, O_2 (solute gas) must be mixed with other gases (balance gases). Nitrogen (N_2) is commonly used alone for this purpose, but some researchers use also argon (Ar). These two gases (N_2 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 (CO_2). It interacts with the bicarbonate buffer in the cell culture medium, stabilizing the pH at about the optimum level (~7.4). CO_2 may be excluded from the gas mixture if the culture is supplemented with a CO_2-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.

O_2 gradient in the microenvironment of the cell

In order to be available for cells in culture, O_2 molecules must be transferred from the gas phase (atmospheric environment) to the liquid phase (culture medium). The rate and extent to which O_2 equilibrium is reached in a medium is a function of the surrounding O_2 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 O_2 detector.
[3] Blank fields represent information lacking from the supplier.

clean benches, under ambient atmosphere, means that excess O_2 is dissolved in the medium. There is thus a lag period after inoculation of the cultures prior to the attainment of O_2 equilibrium in the experimental apparatus. A better practice would be to use a medium that was previously kept at the desired O_2 level prior to culturing (Wion *et al.*, 2011). In addition, the O_2 remaining inside the culture-ware, after culture preparation, requires several hours to reach equilibrium with the adjusted O_2 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 O_2 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 O_2 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 O_2 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 O_2 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, O_2 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 O_2. 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 O_2 when the medium passes through.

Given the many oxygenation methods used by different researchers, how these methods affect the O_2 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 O_2 gradients in cellular microenvironments. One important factor to include in such a description would be how the reported O_2 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 O_2 level. For example, Nahmias *et al.*, (2006) showed that fetal bovine serum plays a role in the cellular reaction to O_2 levels. The authors identified a negative effect of serum on O_2-enhanced metabolism of primary rat hepatocytes cultured on an O_2-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 O_2 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/cm^2. 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 O_2 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 O_2 level in the pericellular environment.

Applications of regulated O_2 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 O_2 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 O_2 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 O_2 concentrations, with resultant consequences for human health (Bates 2012).

Conclusion

Cells *in vivo* are exposed to O_2 levels much lower than atmospheric levels due to the gradual decrease of environmental O_2 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 O_2 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 O_2 level adjustment in primary cell cultures, regulation of pericellular O_2 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.

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