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Cell Handling and Culture Under Controlled Oxygen Concentration

Satoru Kaneko and Kiyoshi Takamatsu

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1. Introduction

The term “cell culture” refers to the *in vitro* proliferation of cells and tissues and involves the use of either primary cells typically having a finite life span in culture or continuous cell lines that are abnormal and immortalized. In the last several decades, efforts have been mainly focused on the sub-culturing of established cell lines and on optimizing culture conditions, including selection of appropriate culture medium, in order to achieve rapid cell growth. The conventional CO₂ incubator only provides minimum requirements for keeping cells alive in a culture environment; pH and temperature are held at 7.4 and 37°C, respectively, and sub-saturated humidity is maintained to avoid evaporative condensation of the culture medium. To avoid contamination of the culture medium from aerosol bacteria, the conventional clean bench removes particles from the working atmosphere by continuous displacement of ambient air that passes through a high-efficiency particle (HEPA) filter, whereas the medium exposed to ambient air quickly discharges dissolved CO₂. *In vivo*, the pH of blood is strictly maintained at ~ pH 7.4 by means of physiological buffer systems, whereas the pH of culture medium mainly depends on a sodium bicarbonate/CO₂ buffer, which is adjusted to a pH of 7.4 in an atmosphere of 5% CO₂; the pK_a is 6.1, and the buffering capacity at pH 7.4 is very weak. The pH and temperature are generally uniform in almost all organs, tissues, and cells in living mammals; however, the dissolved oxygen or oxygen partial pressure (PO₂) in various organs and tissues is generally much lower than that in the ambient air (159 mmHg); it decreases to 100 mmHg and 25 mmHg or less in arterial blood and in the periphery, respectively. Although oxygen is essential to produce ATP through the tricarboxylic acid cycle, it is quite toxic at high concentrations [1-3]. Tissues and cells in body fluids are protected from reactive oxygen species (ROS) by multiple physiological anti-oxidant systems, whereas those in artificial culture medium lack an extracellular protective system and are exposed to high levels of O₂. Numerous studies have cited a variety of harmful effects of ROS, such as lipid and protein

peroxidation as well as membrane and DNA damage [4-8]. Although lower PO_2 implies lower production of ROS, it also implies hypoxia that can damage various cellular functions [9, 10]. The recent advances in tissue engineering focus on clinical applications of cultured cells in regenerative medicine. When primary cells and stem cells are retrieved from human tissue, their original physicochemical environments and metabolic features are quite different from each other. Moreover, induction of differentiation stimulates changes in gene expression over a time course, which may affect cellular metabolism. The above-mentioned point of view suggests that ranges in cell viability, in terms of PO_2 and tolerance for ROS (which need to be controlled for normal proliferation and prevention of malignant transformation), may be quite narrow for primary cells in comparison to cell lines. The stringent control of oxygen during bioprocessing is undoubtedly important; however, the exact influence of PO_2 and ROS is not completely understood because past experimental data have been obtained by using conventional culture apparatuses or their improved models. Although the performance of apparatuses has already been proven by sub-culturing of established cell lines, they have not been designed for stringent control of oxygen throughout the culture period. Every time the incubator door is opened, the O_2 environment is quickly lost and requires a long time to recover. To clarify the net influence of PO_2 and ROS, it is essential to develop advanced equipment that can provide a stringent control of oxygen around the pericellular environment throughout the culture period.

2. Delivery of human babies by assisted reproductive technology: Elementary regenerative medicine involving the transplantation of cultured embryos

In the past 30 years, researchers have made significant progress in the field of clinical reproductive medicine through assisted reproductive technology (ART). In 1978, the first human baby was born through in vitro fertilization-embryo transfer. To date, more than a million children have already been born with the help of ART. Fertilization through intracytoplasmic sperm injection (ICSI) [11] is becoming increasingly popular, and it now accounts for more than half of clinical ART cases worldwide. ART is the first example of large-scale clinical application of regenerative medicine to cultured human stem cells, which involves in vitro fertilization of gametes (primary stem cells) and subsequent culture of early embryos up to the blastocyst stage, followed by transfer into the uterus. Some recent cohort studies could not deny the possibility of birth defects in babies who were delivered as a consequence of ART [12-14], although ART is recognized as an elementary clinical regenerative medicine that makes use of native stem cells. We point out two major issues in this regard: one is quality control of the gametes, and the other is quality assurance of the culture environments. It is well known that human ejaculate contains a heterogeneous sperm population that possesses a variety of abnormalities. ICSI is a technique mainly used in male infertility, which occurs as a result of dysfunction of spermatogenesis and is accompanied by various functional deteriorations in the sperm. Nuclear damage to human sperm, in particular, DNA fragmentation as a consequence of double-strand breaks, has attracted attention. If a sperm with damaged DNA is incorporated into the embryonic

genome, it may lead to sperm-derived chromosomal aberrations [15], which may in turn result in higher miscarriage rates [16] and an increased risk of pregnancy loss [17]. The resultant aberrations can also be potentially inherited through the germ line by future generations [18-20]. Several studies have reported that the rate of DNA-damaged sperm increases in infertile men with poor semen quality, who are the primary candidates for ICSI [21]. Although the techniques in clinical ICSI are well established, the sperm is selected merely based on motility and gross morphology, as observed under a microscope, and there are no validated methods to address and assure sperm nuclear DNA integrity.

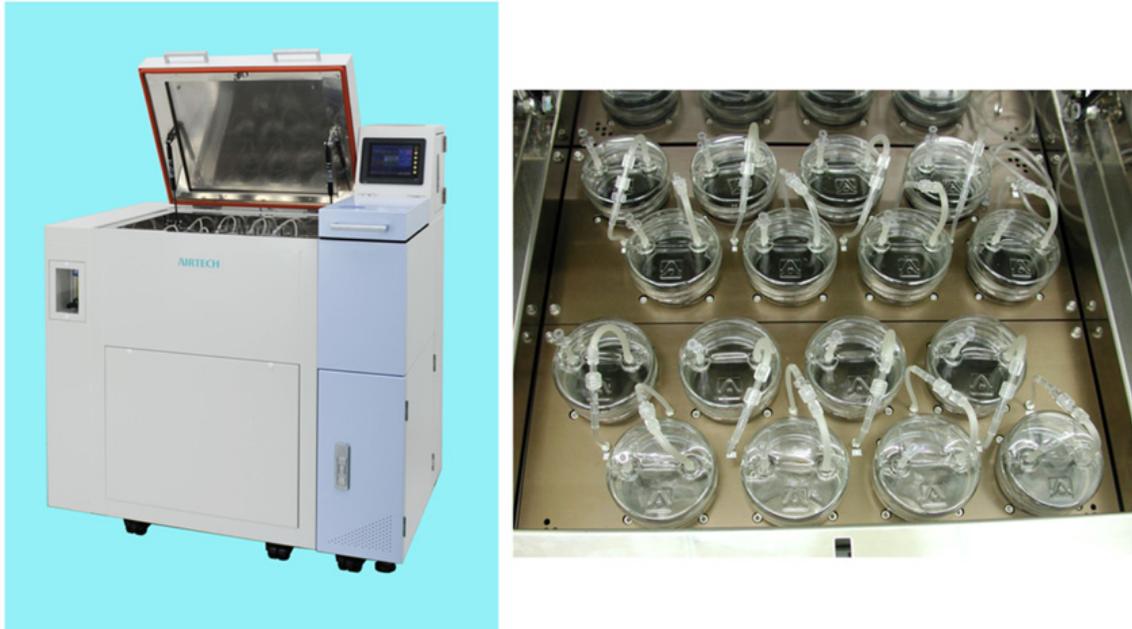
There is a concern about higher malformations resulting from ICSI cycles, due to the possibility of iatrogenic transmission of genetic abnormalities to the offspring [14, 22, 23]. Studies comparing ART cycles and natural births suggest that infants conceived by IVF / ICSI techniques have three times a risk of a congenital heart defect [24] as well as a higher risk of autosomal and gonosomal aneuploidies [25]. It still remains unclear whether culture environments provided by conventional culture apparatus or their improved models have been responsible for the results of various cohort analyses. In general, we have to consider the heterogeneity of the cell population at the start of culture as well as some transformation during the culture. The lumen of the fallopian tube, where the oocyte fertilizes with the sperm, shows very low PO_2 [26], contrary to the endometrium at the implantation phase, which shows thickening with increased blood flow; thus, the implanting blastocyst is exposed to higher PO_2 . During this one-week trip in the oviduct, the embryo undergoes early development in a PO_2 gradient. To determine optimal physicochemical environment for primary and stem cells, including the embryo, one has to pay attention to complicated cross-interactions between the atmosphere, especially PO_2 , and the composition of culture medium: ATP production is influenced by peri- and intra-cellular PO_2 as well as energy sources in the medium. Even if PO_2 is kept low during cell culture, the handling of cells in a clean bench is critical, while temperature, PO_2 , and pH of the medium are dramatically changed. Tolerances to such parameters varies quite differently among cell types. For example, some neuronal cells have a low threshold for oxygen toxicity, and exposure of these cells to ambient air in a clean bench induces apoptosis [3]. Such cells have to be treated in an enclosed space filled with low-oxygen gas mixtures. In contrast, some cells can readily induce apoptosis under low PO_2 conditions [9]. It is well-known that long-term subculture of cell lines induces some genetic transformations. Some researchers [27-31] have proposed that this phenotypic variability might originate from epigenetic alterations, and the methylation profiles of stem cell lines are fundamentally changed during subculture, thus complicating their use in basic and clinical research. Several reports have also discussed the epigenetics of early development [32] and the genetic and epigenetic features of children delivered through ICSI [33]. Kohoda et al. suggested that ICSI induces transcriptome perturbation [34]. To ensure the reliability of clinical embryo cultures, or in general terms, clinical cell cultures, as a premise for human implantation, we have to recognize the complicated cross-interactions of gas phase with composition of the culture medium, cell features, and their heterogeneity with regard to genetic and epigenetic regulation. Numerous reports have emphasized that reducing PO_2 during in vitro cultures increases the proportion of blastocyst formation in mice [35-37], hamsters [38], sheep [39], and cattle [40].

Other studies found no clear effect in mice [41]. As mentioned above, PO_2 and ROS might be essential parameters at least in early embryogenesis [42]. The discrepancies in the results of the above-mentioned studies may be partially explained by differences in culture hardware as well as culture methods: for example, oxygen tension in droplets of medium under oil will be less than those without an oil overlay [43]. Adding EDTA to culture medium increased the proportions of mouse [41, 44] and cattle [40] embryos that developed to blastocysts. Chelating transition metals such as zinc, iron, and copper may prevent chemical reactions that generate harmful oxygen radicals [45]. Because oviductal oxygen tension is less than atmospheric levels [26], mammalian embryos may be protected from oxidative stress in vivo in part by a relatively low oxygen tension in the oviduct [46]. The influence of low PO_2 and hypoxic culture conditions on some cellular functions has also been studied in somatic cells. When BeWo cells, an in vitro model of human trophoblasts, were cultured in 2% O_2 , reverse-transcriptase polymerase chain reaction (RT-PCR) indicated increased transcription of the organic cation transporter (OCTN2) gene compared to that observed under 20% O_2 [47]. Hirao et al. ([48] observed that MC3T3-E1 cells and calvariae from 4-day-old mice cultured in 5% or 20% O_2 conditions showed osteoblastic differentiation and subsequent transformation to osteocytes, which was promoted by low PO_2 . The importance of a lower PO_2 environment was a cited factor; however, excessively low PO_2 are also important to consider for cellular growth, differentiation, gene expression, phenotype manipulation, epigenetics, and moreover, for survival. We consider it essential to determine the narrow range between hyperoxia and hypoxia, but not to overestimate the benefit of lower PO_2 .

3. Individual cell culture systems in a disposable capsule with controlled atmosphere

As will be described later (Figs. 7 and 8), established cell lines that adapt to 5.0% CO_2 -air often tolerate prolonged changes in pH and PO_2 . In contrast, clinical cultures of primary and stem cells used for human transplantation demands rigorous duplication of in vivo environments because it is of prime importance for maintaining normality or to minimize phenotypic changes within the cells. We have previously established an individual cell culture system that emphasizes the precise control of oxygen concentration and quick recovery from disturbances (Figs. 1 and 2) [49]. As shown in Fig. 1, the culture bath has an aluminum block with 16 wells for heat storage, and the block and inner space are kept at 37.0 °C by a temperature sensor. The apparatus is first used as a multivariate screening system for the simultaneous determination of the narrow range between hyperoxia and hypoxia and for designing the optimal formula corresponding to the gas phase. This system can provide up to 16 types of different premixed gases into each capsule individually. The commonly used infrared CO_2 sensor and the Galvanic current O_2 sensor devices have sufficient sensitivity and undergo scheduled calibrations to maintain accuracy assurance. When a small amount of gas is infused for fine control of the gas phase, static diffusion causes an inhomogeneous gas concentration in the chamber, and the display values are often similar to those around the sensors. We therefore used pre-mixed gases and a small capsule for precise control of O_2 concentration. Pure O_2 , CO_2 , and N_2 gases were mixed according to their weight base molar ratios and compressed in the gas canister.. The following gases were used

for cell culture experiments: 2.0% O₂, 5.0% CO₂, and 93% N₂ as an example of hypoxic culture. For purging the capsule, 5.0% CO₂ and 95% N₂ were used. The gas compositions were measured using gas chromatography, according to the pre-shipment review.



Sixteen small capsules for individual cultures were placed in a well of an aluminum dry block.

Figure 1. Individual cell culture apparatus

A



B



A bench top was covered with an acrylic chamber to prevent leakage of 5.0% CO₂-air. Photograph A shows the system equipped with a built-in microscope, personal computer, monitor, and printer. Photograph B shows the apparatus for general handling of cultures without microscopic observation. A minimal volume of internal space is preferable for precision control of the gas phase.

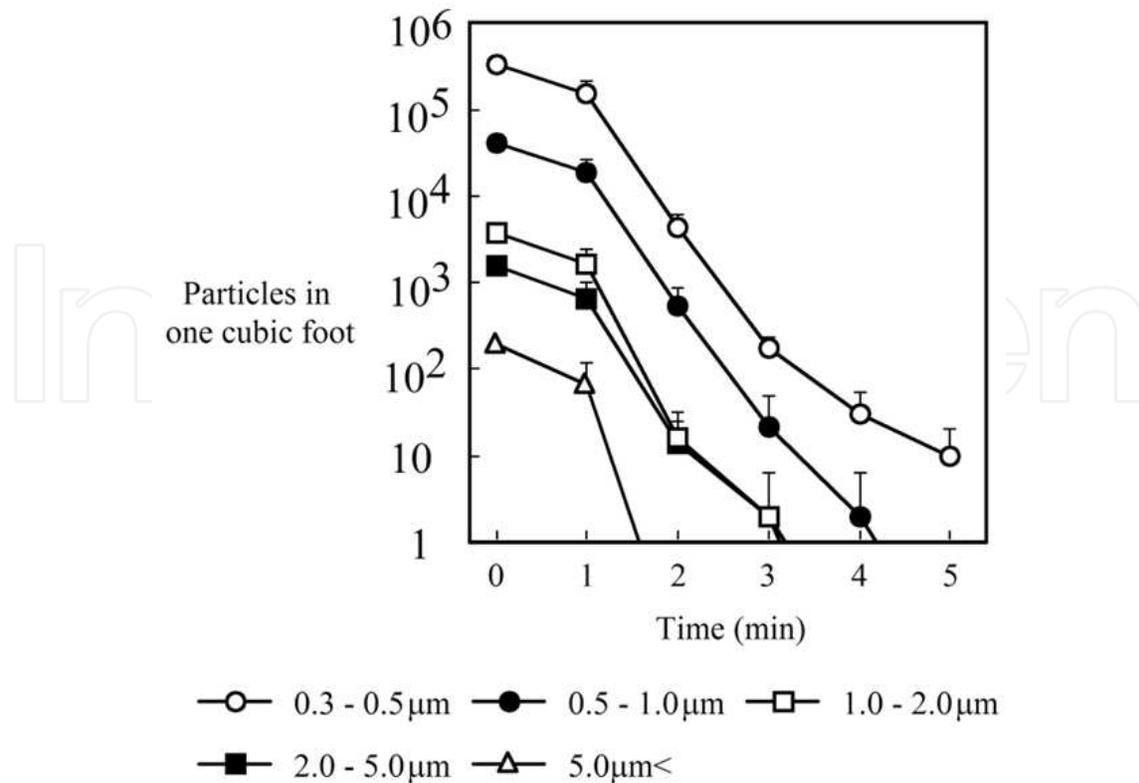
Figure 2. 5.0% CO₂-air circulation clean bench

The degree of cleanliness of air was defined by a “cleanliness class”, which is specified by the number of particles of a size $0.5\ \mu\text{m}$ or over in one cubic feet of air. For instance, a cleanliness class of 100 is interpreted as less than 100 particles in one cubic feet of air. The simultaneous measurements of particle size and number were performed using a light-scattering particle counter. The intake air stream was first passed through a high-intensity laser beam. As a result, the particles in the sample caused light scattering, and their numbers and intensities were detected. Room air often shows a cleanliness class of 10^6 – 10^5 , and the aim of a conventional clean bench is to provide a low-dust environment below a cleanliness class of 10^2 . The cell handling is, however, performed in ambient air, allowing temperature decrease, dissolution of O_2 , and pH change by removal of CO_2 . We newly developed the 5.0% CO_2 -air circulation clean benches with or without a built-in microscope (Fig. 2). The bench top was covered with an acrylic chamber to prevent leakage of the ambient atmosphere, with the set-up resembling an infant incubator. Pure CO_2 was infused with the aid of a gas sensor control to maintain 5.0% CO_2 -air. The bench top and the ambient temperature were kept at 37°C and 30°C – 34°C by temperature control (Fig. 2). In addition, a small chamber was set on the bench top, so that if cells could not tolerate 5.0% CO_2 -air for more than a few minutes, they were isolated in the chamber, and the humidified culture gas was supplied. If the bench top was contaminated with some infectious material such as body fluid, it was merely wiped off. In the newly developed system, a cover shield was placed on the bench top, and a disposable clear film was set and discarded at each operation (Fig. 3). Although the conventional clean bench filtered fresh air only once, the newly developed system circulated the enclosed 5.0% CO_2 -air through a HEPA filter every 24 sec. Before starting the filtration process, the cleanliness class was found to be 10^5 ; however, a cleanliness class of 1 was readily achieved by repeated filtration within 5 min (Fig. 4). Furthermore, particles within the size range of $0.3\ \mu\text{m}$ – $0.5\ \mu\text{m}$ were reduced to less than 100 within 5 min.



In the newly developed system, a bench top was shielded with a clear film for infection control.

Figure 3. Disposable cover film on bench top



Measurements were performed at five time points on a bench. Values are represented as mean \pm standard error.

Figure 4. Change in cleanliness class after starting filters

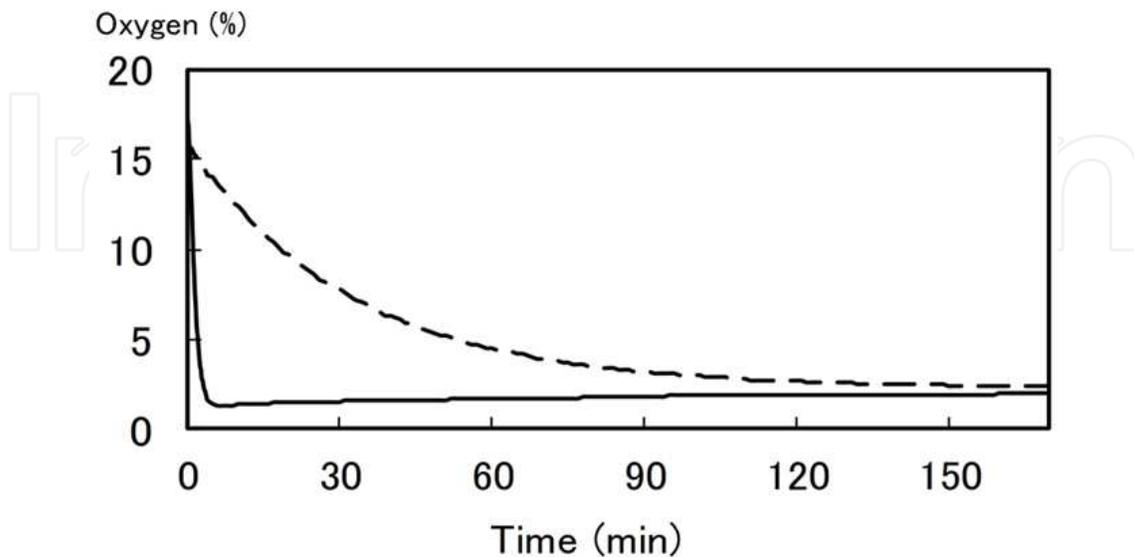
The conventional CO_2 incubator has a structural problem when it comes to achieving a stable hypoxic environment. As summarized in Table 1, whenever the door is opened, a large amount of ambient air intrudes, and the reduced CO_2 can be readily recovered by infusion of pure gas; however, it took more than 30 min to exclude O_2 by flushing with N_2 . Thus, we developed a disposable small capsule to control the gas phase, especially for hypoxic tissue culture (Fig. 5). A 500-ml plastic capsule containing 220 ml of the gas buffer solution (20 mM H, 25 mM NaHCO_3 , and 0.05% Phenol Red) was used as the CO_2 incubator. First, it was equilibrated by ventilation of the pre-mixed culture gas (10 ml/min) for at least overnight. Following gas equilibration, the pH was adjusted to 7.4 ± 0.05 , and the O_2 concentration was measured using a Galvanic current O_2 sensor. Coexistence of a large amount of gas buffer solution, which serves the same function as the culture medium in terms of gas equilibration, stabilizes the physicochemical environment by functioning as a heat storage and gas pool. Inflow of air when the cap is opened should be excluded as soon as possible. To achieve this, the anoxic purging gas (5.0% CO_2 and 95% N_2) was flushed (500 ml/min) just after closing the cap. As a consequence, the oxygen level returned to 2.0% within 4 min, after which the gas supply was changed automatically to culture gas, which was infused (10 ml/min) continuously to maintain positive pressure (Fig. 6). If the gas purging process was omitted, it took 120 min until recovery, despite the inner space volume being only 280 ml (Fig. 6). This fact suggested that the void volume of the culture capsule should be minimized as much as possible, and coexistence of the gas buffer enhance the stability of the gas phase in the culture environment. In this system, gas control through a CO_2 sensor was not necessary, and we also did not need to consider the improper gas control caused by sensor

deterioration. Gas equilibration of each capsule was roughly estimated by checking the color of phenol red in the gas buffer (Fig. 5), and the precision control of the culture environment was monitored by measuring the temperature and pH of the gas buffer. Although simultaneous culturing of multiple tissues is usually possible in a single CO₂ incubator, the present method allows the culturing of individual tissues in disposable capsules. The system also has additional advantages in that it allows easy and error-free identification of dishes and avoids disturbances in culture conditions when the door of the unit is opened.



A maximum of five culture dishes (6.0 cm in diameter) can be placed on the tray of the stainless stand. The gas buffer is placed at the bottom. Two tubes protruding from the cap are inlets and outlets for gas. In-line gas filters (pore size, 0.22 μm) are placed at the inlets and outlets.

Figure 5. Use of disposable capsules for individual cultures with precise control of oxygen concentration and quick recovery from disturbances in culture conditions



: purge with 5.0% CO₂ and 95% N₂; ----- : without purging, supply of culture gas only

Figure 6. Effect of gas purging on O₂ concentration recovery in capsule

Period of open door (sec)	0	10	20	30
CO ₂ (%)	5.0%	5.4%	5.4%	5.3%
Time require for recovery (min)	-	2	2	2
O ₂ (%)	2.1%	13.7%	18.4	18.2%
Time require for recovery (min)	-	30	34	35

The door of a conventional incubator (chamber volume: 30 L) was opened for 10, 20, and 30 sec, and the values on the display unit of the built-in device were observed. The durations required for the recovery were recorded.

Table 1. Time requirement for recovery of gas phase after door opening

The most common cultureware or vessels are sterile, disposable, and specially treated with polystyrene plastic. The cultureware includes petri dishes, multiwell plates, microtiter plates, roller bottles, and screw-cap flasks. All cultureware is equipped with lids or caps to prevent contamination from aerosol bacteria, and these culture vessels are designed to stack. Handling of culture media in conventional or in 5.0% CO₂-air circulation clean benches caused dissolution of oxygen in the media. After the lid was mounted on the culture dish or the cap of the flask was loosely closed, ambient air or 5.0% CO₂-air remained in the inner space of the cultureware. We placed an O₂ sensor on the lid of a culture dish (90 mm diameter, 10 mm height) or on the body of a culture flask (250 ml) to measure the ventilation velocity between the outer and inner spaces of the cultureware. As shown in Fig. 7, when the lid is held in the normal position and placed in the culture gas containing 2.0% O₂, it took more than 40 min for equilibration, despite the inner space volume being only about 60 ml. When the lid was held over the spacers (2 mm and 7 mm in height), the time for equilibration was again shortened to 20 min. If the cells demand a faster velocity of ventilation, a lid made out of gas-permeable materials should be used, or cultureware without lids should be used. A flask with a screw cap has a larger void volume than that of a dish, and, hence, more reliable results were obtained using a flask. The cap of the flask was opened in ambient air and closed loosely. When the flask was placed in the culture-gas environment, the ventilation velocity was found to be extremely low, and it took more than 20 h to attain equilibration (Fig. 8-A). Moreover, the same duration was required for gas leakage, which served as a reversal process (Fig. 8-B). We examined purging of ambient air with anoxic gas in the same manner as described in Fig. 6. A needle was inserted in the cap as a gas injection port, and the flask was capped loosely (Fig. 8). The flushing (500 ml/min) of anoxic gas obviously accelerated the ventilation, and the oxygen level returned to 2.0% within 4 min (Fig. 8-C). This result suggested that the conventional use of a flask with a loose cap, which is subsequently placed in the CO₂ incubator, is unfavorable for primary and stem cells, which were intolerant to prolonged changes in pH and PO₂. An air-tight plastic vessel often suffers from gas leakage through the sealant of wide open-mouthed containers as well as due to the gas permeability of materials. After the cap was closed, the gas phase was recovered by flushing (Fig. 6), and a minimum amount of culture gas (10 ml/min) was supplied constantly (Fig. 9-A) or intermittently (Fig. 9-B) in order to maintain positive pressure. The constant supply of culture gas held PO₂ steady, whereas the intermittent supply caused narrow, wave-like changes due to gas leakage, although their margins of fluctuation were not so much different from each other. The intermittent supply saved gas consumption. The computer-assisted programmable system allowed greater

flexibility to evaluate optimum environmental settings. Fig. 10 shows a time-course model of switching of the gas phase with intermittent gas supply.

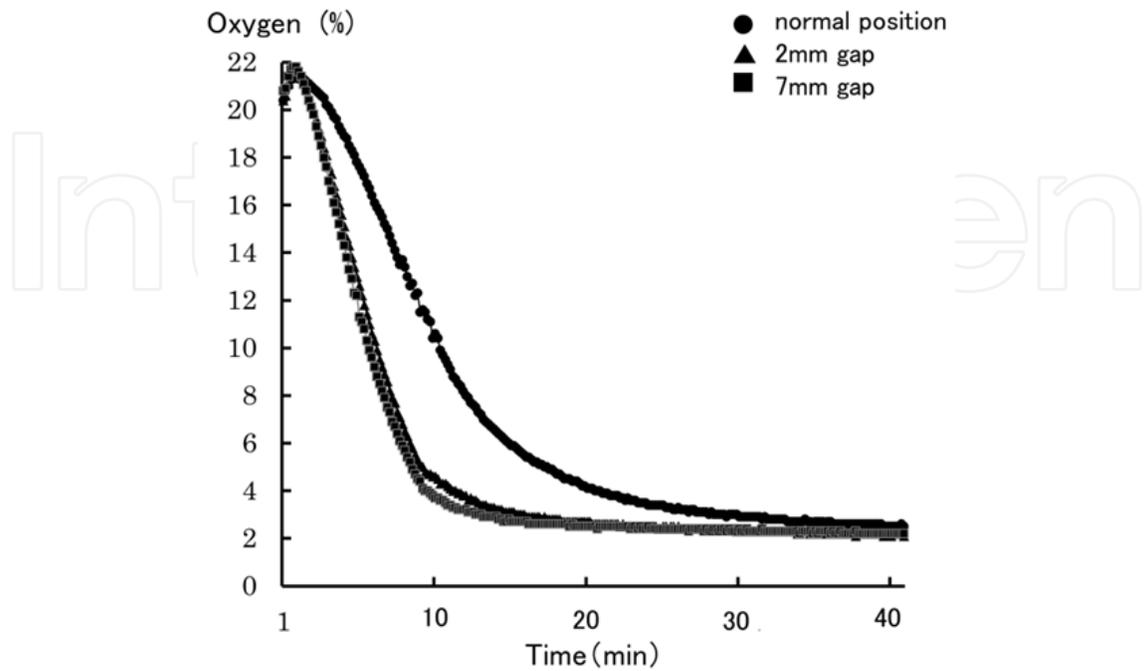
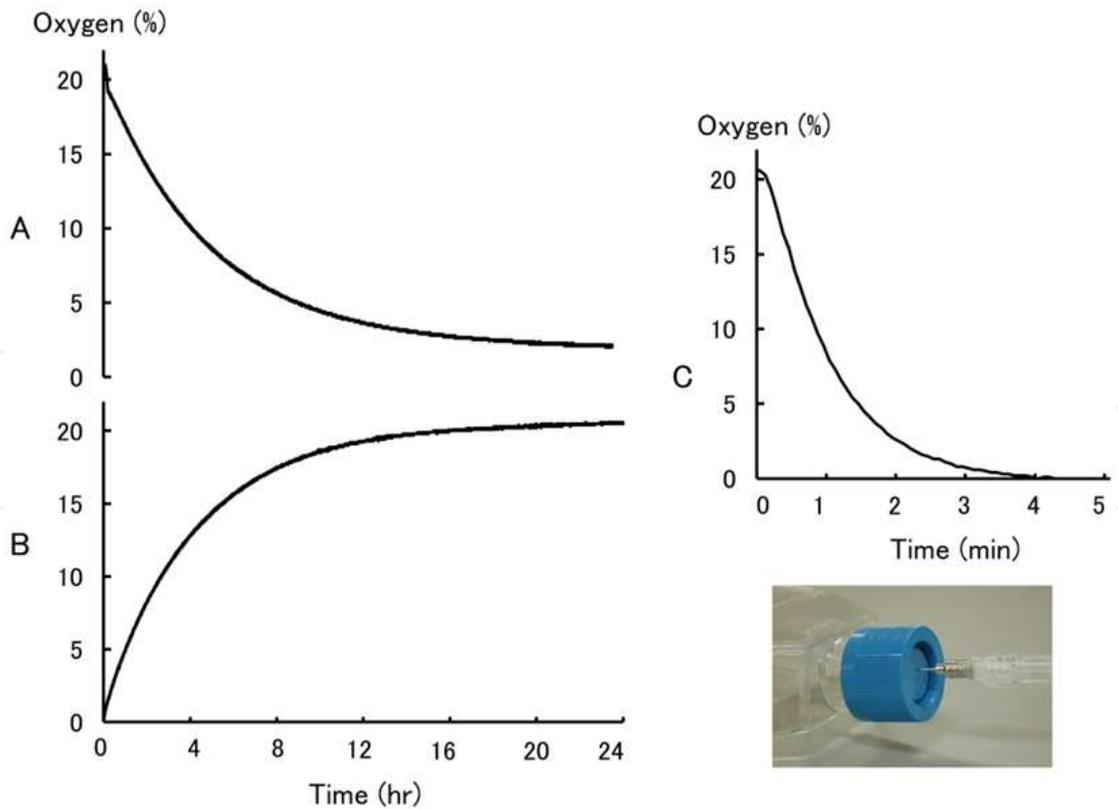
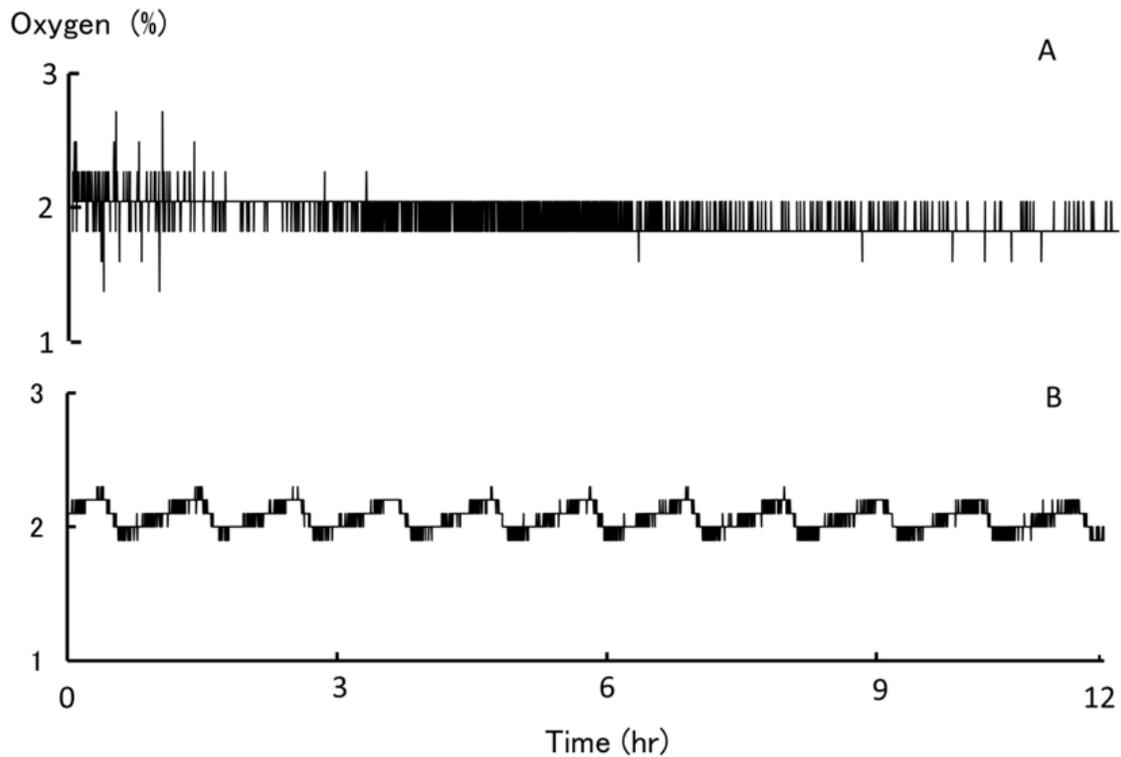


Figure 7. Effect of gap between lid and culture dish on ventilation velocity



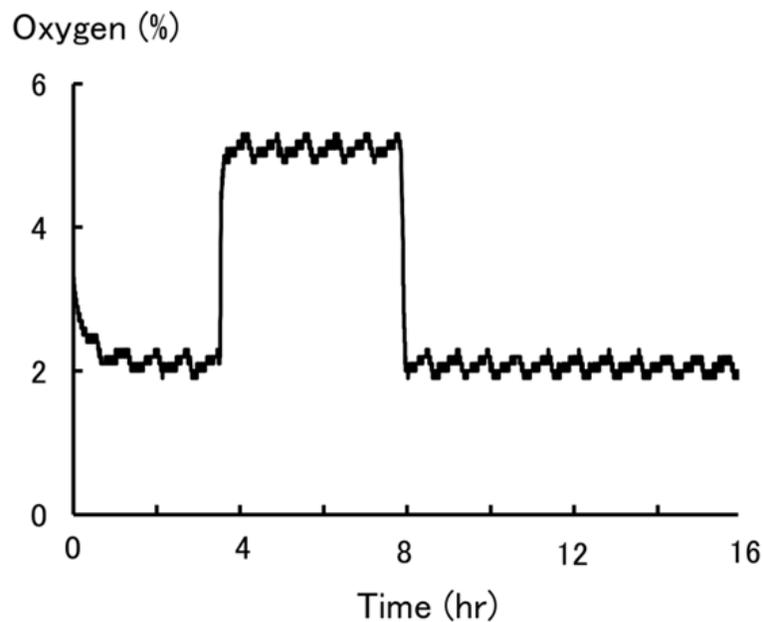
The photograph in the figure shows the experimental set-up of the injection port.

Figure 8. Ventilation velocity of loose cap flask and effects of purging with anoxic gas



The intermittent method constituted a repetitive cycle of a 10-min gas supply followed by a 50-min pause.

Figure 9. Time-course changes in O₂ concentration during constant and intermittent gas supply

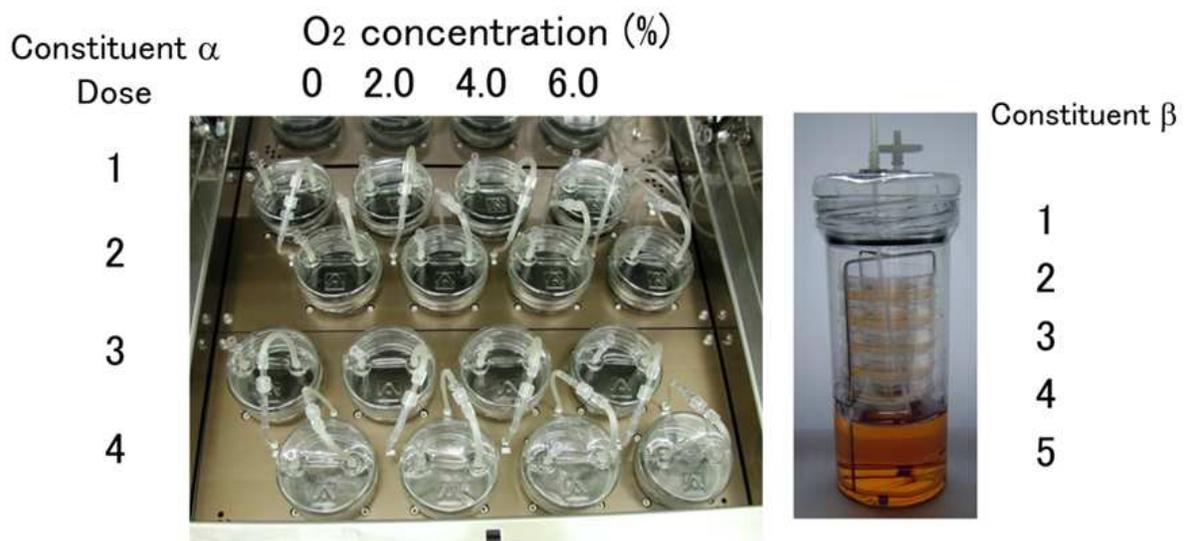


Culture gas was changed from 2.0% O₂ to 5.0% O₂ in mid-course, and then changed back to 2.0% O₂.

Figure 10. A model of computer-assisted programmable intermittent gas supply

The 16 capsules placed in the culture bath (Figs. 1 and 5) were used as a multivariate assessment system to determine the optimal formula corresponding to the narrow range between hyperoxia and hypoxia. Fig. 11 presents the model usage to optimize the

combination of three parameters, namely the four premixed gases with 0% to 6.0% O₂ and the dosage of two constituents. For example, the capsule at the right edge/bottom line the combination of the constituent α , dose 4 and the constituent β , dose 5/6.0% O₂. The formula of widely used media (for example, RPMI and MEM) has been established more than half of a century ago; at that time, the multifaceted pharmacological actions and the concept of genotoxicity of some constituents had not yet been established. Amino acids are often added as supplements in the media, some of which serve as the most abundant neurotransmitters in the brain. Amino acids are responsible for almost all rapid signaling between neurons. For example, glutamate is used as a nitrogen source to promote the syntheses of proteins and nucleic acids, and it is the major excitatory neurotransmitter that is distributed in all regions of the brain ([50]. Inadequate dosing causes glutamate-induced excitotoxicity ([51]. Extracellular ATP ([52], while the decomposed species, adenosine [53], is responsible for calcium channel regulation. It is very important to evaluate whether target cells are pharmacologically sensitive to some of the constituents as well as to impurities and their degraded agents. Moreover, it is important to note that the term “sensitive” includes genotoxicity.



The apparatus can provide a maximum of 16 premixed gases in each capsule, and the combination of a few parameters can be determined simultaneously.

Figure 11. Multivariate assessment of environmental settings

4. Conclusion

To date faster proliferation has often been associated with the optimum culture environment, we have to investigate minutely whether this enhanced proliferation is not caused by genetic transformation or malignant changes or not. The present review dealt with “cell handling and culture under controlled oxygen concentration”. The precision control of oxygen to determine the narrow range between hyperoxia and hypoxia is likely to play an important role in ensuring the safety of cell cultures, especially for primary and stem cells.

Author details

Satoru Kaneko* and Kiyoshi Takamatsu

Reproduction Center, Gynecology, Ichikawa General Hospital, Tokyo Dental College, Sugano, Ichikawa, Chiba, Japan

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* Corresponding Author

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