Original Article

Individual Tissue Culture System in a Disposable Capsule with Hypoxic Atmosphere

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Abstract

In the present study, a novel system for the hypoxic culture of individual tissues was established for the subculturing of cell lines for research as well as for clinical culture of primary cells. To provide a hypoxic environment throughout the process of tissue handling and culture, we designed a clean bench with CO₂ gas circulation and a hypoxic culture incubator containing disposable capsules. The bench top was covered with an acrylic chamber, and an atmosphere of 5.0% CO₂-air was maintained using a sensor control. The cleanliness class of the chamber could easily be improved to 1 within 5 min of circulative filtration, even though it was found to be 10⁵ before the unit was operated. Gas buffer solution (220 ml of 20 mM HEPES, 25 mM NaHCO₃, pH 7.4) placed in a 500-ml plastic capsule in the unit stabilized the culture environment by functioning as a heat storage and gas pool. The inflow of air that occurred by the cap of the capsule was opened was excluded by the infusion of purging gas (5.0% CO₂ and 95% N₂); the O₂ level returned to 2.0% within 4 min, after which the gas supply automatically switched to the culture gas (2.0% O₂, 5.0% CO₂, and 93% N₂). If this purging process was omitted, restoration of the O₂ level required 120 min, even though the inner volume was only 280 ml.

Keywords: individual tissue culture, gas circulation clean bench, hypoxic culture, disposable capsule

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Introduction

Tissue culture plays an important role in cell biology, and it is mainly focused towards the subculturing of established cell lines. Culture equipments such as the clean bench and CO₂ incubator have been widely employed, and they provide the appropriate settings for the handling of tissues by supplying filtered fresh air and an atmosphere with saturated humidity and 5.0% CO₂, respectively. It is well known that the oxygen tension in the periphery is substantially lower than that of fresh air. Only some mutant variants that tolerate the given culture conditions such as the atmosphere and culture medium can adapt to long-term subculturing. Although media compositions have been discussed in detail, few efforts have been channeled towards ensuring that the oxygen tension at the original growth environments remains unchanged. The development of a hypoxic culture system will enable the subculturing of many more malignant tissues than what is currently possible. Recent advances in regenerative medicine require the culturing of primary cells or

stem cells, and advances with regard to transplantation in humans demand more precise duplication of the original growth environments. Recent reports have indicated the influence of hypoxic culture on some cellular functions. BeWo cells - an in vitro model of human trophoblasts - were cultured in 2% O₂, and RT-PCR conducted after the culture indicated that transcription of the organic cation transporter OCTN2 was higher than that after culture in 20% O₂¹⁾. Hirao et al.²⁾ observed that when MC3T3-E1 cells and calvariae from 4-day-old mice were cultured in conditions of 20% or 5% O₂, osteoblastic differentiation and the subsequent transformation to osteocytes were promoted by low oxygen tension.

Some structures and mechanisms of conventional culture equipments are not designed to maintain low oxygen tension in the environment when tissues are being handled and cultured. The present study established a new hypoxic tissue culture system for the culturing of cell lines and primary cells in research and clinical settings, respectively.

Materials and Methods

Measurements with regard to particles in air: The degree of air cleanliness was defined in terms of "cleanliness classes" that are specified by the number of particles

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of size 0.5 μm or more in 1 cubic foot of air³⁾. For instance, cleanliness class 100 indicates less than 100 particles of the specified size in 1 cubic foot of air. Particle size and number were simultaneously measured using a light-scattering particle counter (KC-03A, Rion, Tokyo, Japan). When an intake air stream is passed through a high-intensity laser beam, the particles in the stream scatter light. The particle sizes were divided across 5 categories in terms of the particle diameter (0.3–0.5 μm , 0.5–1.0 μm , 1.0–2.0 μm , 2.0–5.0 μm , and >5.0 μm), and the number of particles belonging to each category was assessed.

Measurement of temperature and O₂ concentration: The temperature sensor in the device (K470; Techno-Seven, Tokyo, Japan) had a resolution of $\pm 0.02^\circ\text{C}$. The O₂ concentration was measured using a galvanic current sensor.

Preparation of premixed gas and accuracy control: In the present study, we used commercially supplied premixed gases for culture (2.0% O₂, 5.0% CO₂, and 93% N₂) and for purging the capsules (5.0% CO₂ and 95% N₂). Pure O₂, CO₂, and N₂ gases were mixed according to their weights of corresponding molar ratios and filled in a container; in a pre-shipment review, their composition was assessed using gas chromatography. Post-receipt accuracy control was performed as follows: when the gases were aerated in a solution containing 20 mM HEPES, 25 mM NaHCO₃ at 37°C, and the composition was considered accurate if the pH of the solution remained at 7.4 $\pm$ 0.05 after gas equilibration.

Results

1. Clean bench with CO₂ gas circulation

In the present study, a new clean bench with CO₂ gas circulation and a built-in microscope was developed. As shown in Fig. 1, the bench top was covered with an acrylic chamber to prevent leakage of the ambient atmosphere; it resembled an infant incubator. Pure CO₂ was infused using a gas sensor control to maintain the composition at 5.0% CO₂-air, and the temperature was maintained at 30–37°C by using a temperature control (Fig. 2). In addition, if the tissue did not allow exposure to 5.0% CO₂-air, the culture dish was isolated in a small chamber placed on the bench top, and humidified culture gas was supplied to it. While fresh air is filtered only once in the conventional clean bench, the enclosed 5.0% CO₂-air was circulated through HEPA filter every 24 s in the present system. As shown in Fig. 3, the cleanliness class of the air was found to be approximately 4×10^5 before operation of the unit was initiated; particles larger than 0.5 μm were not found, and only 10 particles of size 0.3–0.5 μm were observed in 1 cubic foot of air after 5 min of operation. Repeated filtration easily yielded cleanliness class 1. Thus far, if bench tops were contaminated by some

infectious material such as body fluid, they were merely wiped clean. In the present system, however, a disposable clear film is placed as a covering shield on the bench top, and it is discarded after each operation.

2. Hypoxic culture of individual tissues in disposable capsules

We developed a disposable capsule for hypoxic tissue culture (Fig. 4). A 500-ml plastic capsule containing 220 ml of the gas buffer solution (20 mM HEPES, 25 mM NaHCO₃) was used; it functioned as a heat storage as well as a gas pool. The culture bath had a 16-well aluminum block for heat storage, and the block and the inner space were maintained at 37°C by using a temperature sensor (Fig. 5). First, the gas buffer solution was equilibrated by infusion of a small amount of the culture gas (10 ml/min) at least overnight. When the door was opened for 10 s, a slight temperature change of 0.1°C was observed in the inner space of the capsule, and it was restored within 7 min.

When conducting tissue culture using this capsule, the inflow of air when the cap is opened should be excluded as soon as possible; The purging gas was flushed (500 ml/min) immediately after the cap was closed, and the O₂ level was restored to 2.0% within 4 min; the gas supply automatically switched to the culture gas, which was continuously infused (10 ml/min) to maintain positive pressure (Fig. 6). If this process was omitted, the restoration of the O₂ level required 120 min, even though the inner volume was only 280 ml. In this system, gas control with a CO₂ sensor was unnecessary, and improper control due to the sensor deterioration was not required to be considered. Gas equilibration in each capsule was roughly estimated by the color of phenol red in the gas buffer solution, and the precise control of the culture environments were monitored by measuring the temperature and pH of the gas buffer solution. Although simultaneous culturing of multiple tissues in a single CO₂ incubator is usually possible, the present method that facilitates the culturing of individual tissues in disposable capsules has some advantages: the individual dish can be easily identified without any confusion, and the culture conditions are not disturbed when the door of the unit is opened.

Discussion

The cleanliness class of room air is generally 10^6 – 10^5 . A low-dust environment with a cleanliness class of 10^2 would be provided when the conventional clean bench is operated under the optimum conditions. However, tissues are handled in fresh air, and under these conditions, O₂ may be dissolved in the medium, and the pH could change due to CO₂ removal. The present clean bench with CO₂-air circulation had overcome the above defects, and the functions had been rather similar with the con-

Fig. 1 Clean bench with CO₂ gas circulation

Fig. 2 Temperature of the clean bench
A: fresh air, B: ambient atmosphere, C: bench top

Fig. 3 Change in cleanliness class after beginning of operation
The values are represented as mean ± standard error. They were measured at 5 points on the bench.

Fig. 4 Disposable capsules for hypoxic tissue culture
A maximum of 5 culture dishes (6.0-cm diameter) can be placed on the tray on the stainless steel stand. The gas buffer solution is placed at the bottom of the stand. The 2 tubes protruding from the cap are the gas inlet and outlet.

Fig. 5 Culture bath with a 16-well aluminum block

Fig. 6 Effect of gas purging on the restoration of the O₂ level after the cap is closed
A: purging with 5.0% CO₂ and 95% N₂, B: no purging, but 10 ml/min supply of 2.0% O₂, 5.0% CO₂, and 93% N₂. The arrow indicates gas switching.

ventional CO₂ incubator.

The conventional CO₂ incubator poses a structural problem if hypoxic tissue culture is to be performed. The inflow of fresh air cannot be avoided when the door is opened. The concentration of CO₂ can be restored by infusing pure CO₂ gas. As shown in Fig. 6, it is very difficult to remove O₂ once it enters the chamber, and the tissues may be exposed to a high concentration of O₂. It is well known that the partial pressure of O₂ in fresh air (159 mmHg) is reduced to 100 and 25 mmHg or less, respectively, in arterial blood and at the periphery. When the saturated vapor pressure at 37°C was estimated to be 47 mmHg, those in 5.0% CO₂-air and the culture gas were calculated to be 142 and 14.3 mmHg, respectively. Numerous authors have shown that reactive oxygen species (ROS) exert various harmful effects such as lipid and protein peroxidation and membrane and DNA damage⁴⁻⁷⁾, however, only a few reports have addressed the effects of O₂ tension in culture environments^{1,2,8)}. Tissues and cells in body fluids are protected from O₂ and ROS by physiological antioxidants that are limited in artificial culture media, and an extremely high level of O₂ promotes ROS generation. Since the tolerance of cells to O₂ and ROS differs, the optimum O₂ concentration should be established for each cell line in order to minimize cellular damages and subsequent mutation. In the present system, by changing the composition of the premixed gas, the concentration of O₂ can easily be set, in the range of 0%–95%.

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