Investigation on the Suitable Pressure for the Preservation of Astrocyte

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Abstract. The effects of pressure on the survival rate of astrocytes in growth medium (DMEM) were investigated at room temperature and at 4°C, in an effort to establish the best conditions for the preservation. Survival rate at 4°C was found to be higher than that at room temperature. The survival rate of astrocytes preserved for 4 days at 4°C increased with increasing pressure up to 1.6 MPa, but decreased with increasing pressure above 1.6 MPa. At 10 MPa, all astrocytes died. The survival rate of cultured astrocytes decreased significantly following pressurization for 2 hours and the subsequent preservation for 2 days at atmospheric pressure. Therefore, it is necessary to maintain pressure when preserving astrocytes. These results indicate that the cells can be stored at 4°C under pressurization without freezing and without adding cryoprotective agents. Moreover, it may be possible to use this procedure as a new preservation method when cryopreservation is impractical.

1. Introduction

Cells dissociated from tissues are often cultured in vitro and are investigated for physiological activity. Such cells can also be used in experiments or as samples for the development and production of medicine. In recent years, scientists have increasingly replaced animal studies with cultured cell experiments in response to public concern over the ethics of animal experimentation [1]. Although passage culture is commonly used, primary culture is an approach that aims to maintain the properties of cells isolated from living tissue. Thus, there is a need for methods of cell preservation that maintain the desired properties at suitable rates in order to perform investigative experiments and comparative studies.

Cryopreservation, in which cell lines are maintained at or near the temperature of liquid nitrogen, is a critical methodology in biological research. However, freezing induces cell death due to intracellular ice formation and chemoosmotic stress, which result in plasma membrane disruption and subsequent necrosis. To prevent ice formation, freezing speed must be controlled using a programmable freezer. As an alternative method, numerous investigators have cryopreserved cellular systems using cryoprotective agents (i.e., dimethyl sulfoxide, glycerol or sugar) in an extracellular-like carrier solution, such as standard cell culture medium, but this approach has a risk of harmful effects due to the cryoprotective agents themselves [2].

On the other hand, high pressure has recently been the subject of much interest in several fields...
[3, 4]. For example, it is known that water does not freeze under a pressure of 200 MPa [5]. In addition, it has been reported tadpoles awake from anesthesia at 150 MPa, and that bacteria can be killed with applied pressure [6, 7]. However, the influence of high pressure on cell preservation is not currently known [8]. We investigated earlier whether cells (astrocytes) can be preserved in a pressurized system, without the formation of ice or addition of cryoprotective agents, at the temperature above 0°C [9]. In this study, we have performed a more detailed analysis of the relationship between pressure and survival rate in order to decide the best conditions for the preservation.

2. Materials and methods

2-1 Cell culture
Astrocytes were obtained from a neonatal rat brain-derived primary culture (CLEA Japan, Inc.) [10]. Astrocytes in DMEM (Dulbecco’s Modified Eagle Medium, Invitrogen) containing 10% FBS (fetal calf serum; Gibco BRL) were incubated at 37°C under 5% CO$_2$ / 95% air for 20–30 days. As living astrocytes adhere to the surface of the culture flask, cells were detached with 0.05% trypsin (Gibco BRL). Detached cells were centrifuged at 800 rpm for 7 min, and were suspended in DMEM. Suspended cells were subjected to filtration (100-µm mesh), and the number of astrocytes was counted under a microscope. The density used in this experiment was controlled to 1–3 × 10$^6$ in DMEM (300 µl) [9].

2-2 High pressure treatment
Cells suspended in DMEM (300 µl) were put into a silicon tube (5-mm inner diameter, 7-mm outer diameter) and both sides were sealed with Teflon rods (6-mm diameter, 7-mm length) (Fig. 1). This inner vessel was set in a pressure vessel filled with a pressurizing medium (water) (Fig. 2). The temperature of the high-pressure vessel was kept at 4°C in a constant-temperature water bath. Using a plunger pump (Shimadzu LC-6A), the pressurization rate (0.15–50 MPa min$^{-1}$) was controlled by regulating the flow rate (range: 0.01–9.00 mL min$^{-1}$). Pressure was held constant, after reaching the target pressure, by closing the stop valve. As the silicon tube of the inner vessel is flexible, the pressure applied in the pressure vessel is uniformly transmitted to astrocytes in the inner vessel [9].

Figure 1. Inner vessel, comprising Teflon rods and silicon tube [9].

Figure 2. High-pressure equipment used in this experiment [9].

2-3 Determination of astrocyte survival rate
Live astrocytes adhere to the surface of the culture plate and extend processes. However, dead astrocytes float in medium and have a spherical shape. Therefore, survival rate was evaluated as follows. After pressurization at various levels, astrocytes were removed from the pressure vessel
and were suspended in 3 mL of DMEM, followed by dilution to 1–3 × 10^6 cells 300 µL⁻¹. These astrocytes were dispensed into 3 wells of a 12-well tissue culture plate, and were incubated at 37°C under 5% CO₂ / 95% air. After 2 days, live (adhered to the plate surface) and dead (floating in medium) cells were counted under a microscope (Nikon Eclipse TS100). Survival rate was defined semi-quantitatively as follows:

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\text{Survival rate} (\%) = \frac{\text{number of adhering cells}}{(\text{total number of adhering cells and suspended cells})} \times 100.
\]

3. Results and Discussion

As a typical example, firstly we show photomicrographs of astrocytes incubated for 2 days in a CO₂ incubator after preservation at atmospheric pressure and 1.6 MPa for 4 days at 4°C in Figures 3(a) and 3(b), respectively. Almost all of the astrocytes preserved at atmospheric pressure (Fig. 3(a)) were floating in the medium and had a spherical shape. On the other hand, many of the astrocytes preserved at 1.6 MPa adhered to the surface of the plate (Fig. 3(b)). This indicates that the number of living cells preserved under high pressure was larger when compared to preservation at atmospheric pressure. Previously, we reported that both survival rates at 4°C and room temperature were the same under high pressure of 0.3-0.6 MPa [9]. A further detailed analysis of the relationship between pressure and survival rate performed in this study is presented in Fig. 4. Survival rate is given as a relative value, as described in the Methods section. As shown in Figure 4, the survival rate of astrocytes preserved for 4 days at 4°C increased with increasing pressure up to 1.6 MPa, but decreased with further applied pressure above 1.6 MPa. At 10 MPa, all astrocytes died. The relationship between pressure and survival rate after preservation for 4 days at room temperature is shown in Fig. 5. Astrocytes preserved at pressure (0.3–0.6 MPa) had a survival rate of about 50%, but nearly all of the astrocytes died at pressures above 1.1 MPa. We point out that, in contrast to previous result, the survival rate of cells preserved under high pressure (0.3-0.6 MPa) at 4°C was higher than that preserved under the same pressure at room temperature.

**Figure 3.** Photomicrographs of astrocytes incubated for 2 days in a CO₂ incubator after preservation at atmospheric pressure (a) or 1.6 MPa (b) for 4 days at 4°C.
Moreover, we checked whether it is better to keep the applied pressure with some time for the preservation of astrocytes. We found that the survival rate of cultured astrocytes decreased significantly following pressureization for 2 hours at 1.6 MPa and 4°C and subsequent preservation for 2 days at atmospheric pressure. Therefore, it is necessary to keep pressurizing while preserving astrocytes.

The effects of the pressure on whole organisms, proteins and seeds have been examined widely, and can be summarized as follows [3, 11–15]. Protein subunits are dissociated at 200 MPa [12]. Quaternary enzyme structure is disrupted at less than 200 MPa, and enzymes are deactivated
reversibly at 300–400 MPa [14, 15]. Microbes, including viruses, are killed at about 350 MPa [3, 7]. Proteins are denatured at 400 MPa [3, 15], and irreversible inactivation of enzymes occurs at above 600 MPa [2]. However, at lower pressures of a few MPa (about 1.6 MPa), we succeeded in preserving astrocytes, and the survival rate at 4°C was higher than at room temperature. Finally, from these result, we proposed the suitable condition for the preservation of astrocytes.

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