Transportable, small high-pressure preservation vessel for cells

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Abstract. We have previously reported that the survival rate of astrocytes increases under high-pressure conditions at 4°C. However, pressure vessels generally have numerous problems for use in cell preservation and transportation: (1) they cannot be readily separated from the pressurizing pump in the pressurized state; (2) they are typically heavy and expensive due the use of materials such as stainless steel; and (3) it is difficult to regulate pressurization rate with hand pumps. Therefore, we developed a transportable high-pressure system suitable for cell preservation under high-pressure conditions. This high-pressure vessel has the following characteristics: (1) it can be easily separated from the pressurizing pump due to the use of a cock-type stop valve; (2) it is small and compact, is made of PEEK and weighs less than 200 g; and (3) pressurization rate is regulated by an electric pump instead of a hand pump. Using this transportable high-pressure vessel for cell preservation, we found that astrocytes can survive for 4 days at 1.6 MPa and 4°C.

1. Introduction
Cells can generally be preserved in a frozen state at low temperatures of around -80°C [1]. However, cell injury often occurs due to ice crystal formation [2]. To prevent ice crystal formation, various methods have been reported. The most common method involves the addition of cryoprotective agents such as ethylene glycol and dimethylsulfoxide [3-7], but these agents can cause damage to some cells [8,9]. Although innocuous cryoprotective agents to prevent ice crystal formation have been studied [10], such agents have remained elusive. A recently devised method, vitrification, also effectively prevents ice crystal formation [11-13], but requires advanced technology to achieve.

We previously established a novel cell preservation method that uses high pressure at 4°C and does not require freezing or the addition of cryoprotective agents [14]. Therefore, there is no damage due to ice crystal formation or the use of chemical agents. This method will likely be useful for the preservation of embryonic stem cells (ES) or other cells that are limited by low yields of viable cells following freezing and thawing [15]. However, most pressure vessels are associated with various problems for use in cell preservation and transportation: (1) they cannot be readily separated from the pressurizing pump in a pressurized state; (2) they are heavy and expensive because they are made of materials such as stainless steel; and (3) pressurization rate is difficult to regulate with a hand pump. Therefore, we developed a transportable vessel suitable for cell preservation under high-pressure conditions. The effectiveness of this device was also examined based on the survival rate of astrocytes.
2. Methods

Astrocytes were obtained from a neonatal rat brain-derived primary culture (CLEA Japan, Inc). Cells in growth medium, DMEM (Dulbecco’s Modified Eagle Medium; Invitrogen), containing 10% FBS (fetal bovine serum; GIBCO BRL) were incubated at 37°C under 5% CO₂ : 95% air for 20-30 days. Living astrocytes adhere to the surface of the flask and extend processes. Cells were thus exfoliated with 0.05% trypsin (GIBCO BRL) from the surface of the flask and were treated in a centrifuge (0.8×1000 rpm, 7 min). The cell suspension was then passed through a 100-µm filter, and the filtrate was collected. Cells were counted under a microscope, and the cell density was found to be about 2.0×10⁶ cells/300 µL. The temperature of the high-pressure vessel was maintained at 4°C using a constant-temperature water bath, and the pressure was maintained at 1.6 MPa or 0.1 MPa (control). Live astrocytes adhere to the dish surface and extend processes (Figure 1a), but dead astrocytes float in the medium and take on a spherical shape (Figure 1b). Therefore, survival rate was evaluated as follows: after storage under 1.6 MPa for 4 days at 4°C, cells in DMEM (Invitrogen) containing 10% FBS (GIBCO BRL) were incubated at 37°C under 5% CO₂ : 95% air on nine-well plates. After 2 days, the number of living (adhering to the plate surface) and dead (floating in medium) was counted with a microscope (Nikon Eclipse TS100). Survival rate was defined 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.
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3. Results and Discussion

The high-pressure equipment is largely composed of two parts. One is the pressure apparatus (pressurizing pump and pressure gauge) and the other is the high-pressure vessel, as shown in Figure 2. The inner vessel is placed inside the high-pressure vessel. Cells are placed in the inner vessel to prevent contamination, and are pressurized by hydrostatic pressure.

Figure 3 shows the high-pressure vessel, which is composed of a stop valve and cylinder-type vessel. High-pressure vessels are generally made of stainless steel, which is heavy and expensive. We decided to use PEEK (polyether ether ketone) resin due its light weight, cost and ease of handling. The length of the cylinder-type vessel is 90 mm, and the diameter is 30 mm. In the center, there is a hole to allow insertion of the inner vessel. The length of the hole is 60 mm, and the diameter is 10 mm. As a result of using PEEK, the gross weight of the pressure vessel, including the stop valve, is 200 g, and the cost is about one-tenth that using stainless steel. The pressure inside the vessel is maintained by closing the stop valve after pressurization. No pressure leakage was observed after 4 days at 15 MPa.
There are two problems that occur if cells are placed directly in the high-pressure vessel: (1) the cells are easily contaminated; and (2) the high-pressure vessel becomes contaminated, thus increasing the risk of infection. Therefore, cells have to be placed in the inner vessel in order to prevent these problems. Figure 4(a) shows the inner vessel, which is composed of teflon plugs and a silicon tube. Cells are pressurized uniformly by hydrostatic pressure because the silicon tube is soft. The inner vessel can be sterilized with an autoclave.

Empirically, it is known that the survival rate of cells is higher in the cell-condensed state. In the inner vessel, cells are condensed by gravity. Because the cells cannot condense if the vessel is laid down, the high-pressure vessel should be set perpendicularly (Figure 2b). In addition, a cone-shaped hole is cut into the bottom teflon plug to allow for greater condensing efficiency (Figure 4b).

When a hand pump is used, regulating the pressurization rate is difficult, and it is not possible to gradually increase pressure. To overcome these issues, we used a plunger pump (Shimadzu LC9A), which allows the pressurization rate to be controlled by controlling flow rate (0.01 to 9.00 mL/min range).

Figure 5(a) shows a photomicrograph of astrocytes cultivated for 2 days in a CO\textsubscript{2} incubator at 37°C under after pressurized storage at 1.6 MPa for 4 days at 4°C. On the other hand, Figure 5(b) shows a photomicrograph of astrocytes cultivated for 2 days in a CO\textsubscript{2} incubator after storage at atmospheric pressure for 4 days at 4°C. The survival rates were 80% for astrocytes stored under 1.6 MPa for 4 days at 4°C and 0% for those stored at atmosphere pressure for 4 days at 4°C. When the teflon plug having the cone-shaped hole was used, the survival rate was higher when compared to that with the flat teflon plug after storage under 1.6 MPa for 4 days at 4°C.
Thus, high-pressure equipment (light, inexpensive, easy to handle) for cell preservation was produced. In the near future, it will be necessary to examine how survival rate is influenced by vibration during transport.

Figure 5. Photomicrographs of astrocytes cultivated for 2 days in a CO$_2$ incubator at 37°C after storage under 1.6 MPa 4 days at 4°C (a), and astrocytes cultivated for 2 days in a CO$_2$ incubator at 37°C after storage at atmosphere pressure for 4 days at 4°C (b).

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