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EFFECTS OF ADDITIVES AND COOLING RATES ON CRYOPRESERVATION PROCESS OF RAT CORTICAL CELLS

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1 INTRODUCTION

Dissociated cells from animals are widely used in in vitro models to enable the investigation of principal biological mechanisms. The cells are incubated with culture medium immediately after collection. Subsequently, they are maintained under suitable conditions by subculturing and exchanging fresh culture medium. Another suitable technique that can be used to obtain cells for experimental studies is the cryopreservation of dissociated cells. Successful cryopreservation and recovery of primary cell cultures would be valuable for extended and prolonged studies.¹ Cryopreserved neurons would be in demand by industries; for example, high-quality neurons are useful for the screening of new drugs.

There are some important factors that need to be considered when cells are to be frozen. These include the type of cryoprotectant used to inhibit the formation of intra- and extracellular ice and the cooling rate of cells.

Cryoprotectants are added to the culture medium in order to protect cells. Glycerine, the first cryoprotectant to be used, was discovered in the 1940s,² and it was used for the cryopreservation of bovine sperm. More recently, it has been discovered that other substances such as dimethyl sulfoxide (DMSO),³ saccharides,^{4,5} and proteins also function as cryoprotectants.⁶ However, the mechanism of the cryopreservation process in each case remains unclear. In the present study, we compared DMSO and trehalose, which are cryoprotectants of different molecular sizes that may differ in their ability to pass through the cell membrane.

The cooling rate of cells is also an important factor. In slow freezing, the amount of ice that is generated can be controlled by the dehydration of the cell due to the presence of extracellular ice, which results in a higher solute concentration. It is more difficult to freeze cells treated in this manner because the freezing point is depressed at higher solute concentrations. However, under more rapid freezing conditions, ice is generated intracellularly because there is insufficient time for dehydration. Intracellular ice might disrupt cell membranes. Therefore, for cryoprotecting a particular cell with a particular cryoprotectant, there will be a suitable cooling rate.

The cryopreservation technique has been established for some cells.⁷ As a result, these cryopreserved cells have been used in many studies. However, in the case of neurons, the appropriate cryopreservation process has not yet been established because of their complex structure, low mitotic activity, and uncertainties regarding the cryoprotection mechanism. Thus, primary culture cells are used for studying neurons.

Neurons show a spontaneous wave of electrical excitation known as an action potential or nerve impulse, which is characterized by pulses that have an amplitude of several dozen microvolts and a width of a few milliseconds. The impulse carries a message without attenuation from one end of the neuron to the other end at speeds as high as 100 m/s or more. This property is important for evaluating the cryopreservation of these cells. Previously, the nerve impulse has been measured using a single electrode. However, this is not useful for evaluating the effectiveness of cryopreservation of a large number of neurons. Recently, a useful device that can be used to measure many nerve impulses has been developed. The multiple electrode device (MED) system simultaneously measures the nerve impulse of recovered neurons.⁸⁻¹⁰ Using this MED system, we can observe the normal behavior of these cells.

We studied the effect of cryoprotectants and cooling rates on the cryopreservation of rat cortical neurons. We then evaluated the recovering neurons by observing them by phase-contrast microscopy and by measuring their action potentials with the MED system.

2 MATERIALS AND METHODS

Primary rat cortical cells were prepared from neonatal Sprague-Dawley (SD) rats within 3 days of birth (Primary Cell Co., Ltd.). The dissociated cells contained neurons, glial cells, and fibroblasts. These cells were suspended in the culture medium at 1×10^6 cells/ml solution. The materials that were to be tested as cryoprotectants were then added to the medium. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) and 10 vol% fetal bovine serum (FBS). Control dissociated neurons that had not been treated with the cryopreservation were cultured so that they could be compared with the cryopreserved neurons.

Three types of cryoprotectants at various concentrations were selected: DMSO (5–20 wt%), trehalose (3–17 wt%), and a mixture of trehalose (10 wt%) and methanol (8 wt%). A mixture of methanol and trehalose has been reported to be effective for the cryopreservation of salmon sperm.¹¹ Therefore, this mixture was also used to check its effectiveness in the cryopreservation of neurons.

To study the effect of the cooling rate, we varied the cooling rate of cell suspensions from slow (10 K/min) to rapid (10^2 K/min). The slow cooling-rate experiments were performed by placing the cryotube containing the sample into a freezer (at 193 K). The rapid cooling-rate experiments were performed by placing this tube inside liquid nitrogen (at 77 K). The storage temperature was 193 K for the slow cooling-rate experiments and 77 K for the rapid cooling-rate ones. At temperatures below 193 K, the growth of ice becomes slow. Therefore, we thought that the results will not change over this short storage period.

After storing the samples at each temperature for 1 week, they were rapidly thawed in a warm bath (at 310 K). They were then cultured on poly-L-lysine-coated 24-well plates or the MED probe, which is a plate used to measure nerve impulses, in an incubator (humidified atmosphere including 5% CO₂ at 310 K) after replacing the cryoprotectant with the culture medium. The recovering neurons were first examined by phase-contrast microscopy and compared with control samples. The presence of neurons

was also checked by immunofluorescence staining of microtubule-associated protein 2 (MAP2), which is a neuron-specific marker. Apart from this, the number of neurons present was counted by microscopic observation.

To verify the successful recovery of cells, we measured the nerve impulse using the MED 64 system. Neurons were cultured on the MED probe on which 64 electrodes are located. Thus, the nerve impulse can be measured more easily in this system than in a single electrode system.

3 RESULTS AND DISCUSSION

3.1 Control cultured neurons

Initially, we cultured the control neurons without cryopreservation. Neurons cultured on a poly-L-lysine-coated 24-well plate were examined by microscopy and immunofluorescence staining using MAP2 (Figures 1,2). Arrows indicate living neurons.

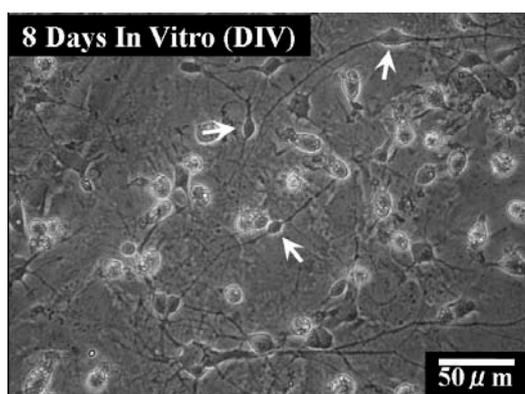


Figure 1 Control cultured neurons that were not cryopreserved. (phase contrast image).

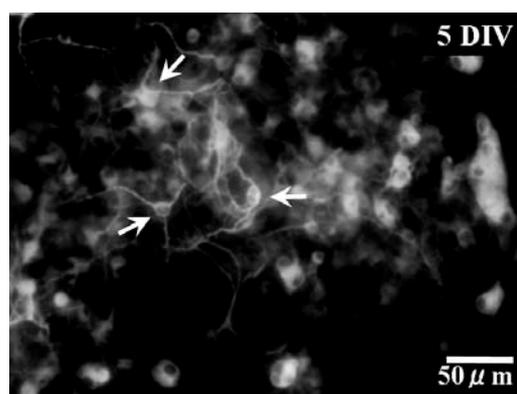


Figure 2 Control cultured neurons stained with MAP2.

Figure 3 shows the nerve impulse measured with the MED 64 system. (amplitude: $28 \mu\text{V}$, width of pulse: 4 ms) In the present study, this was defined as the general nerve impulse of neurons. The MED 64 system is useful for evaluating neuronal function.

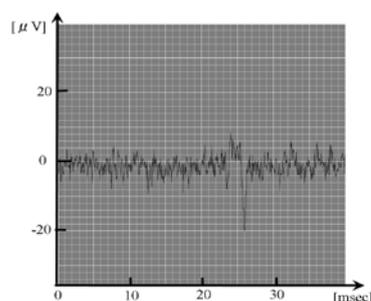


Figure 3 Nerve impulse of control neurons measured with the MED 64 system.

In order to check the effectiveness of the cryoprotectants, neurons were cryopreserved with regular culture medium (without cryoprotectants). Recovering cells were not observed in microscopic observations when cryopreservation was carried out using the same experimental protocol (Figure 4). In case of cryopreservation in the absence of cryoprotectants, the ice around the cells disrupts the cell membrane.

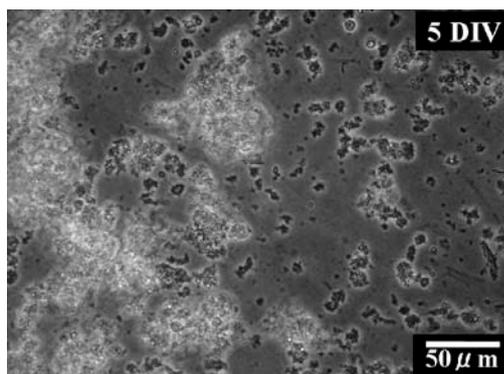


Figure 4 Recovering cells (after cryopreservation in the absence of cryoprotectants).

3.2 Cryopreservation with DMSO

Cryopreservation with 5–10 wt% DMSO showed that recovering neurons were present; this was determined by microscopic observations and immunofluorescence staining using MAP2 (Figures 5,6). White arrows show living neurons, and black arrows indicate dead neurons.

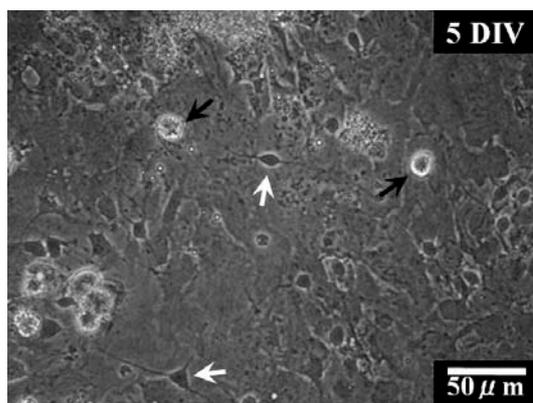


Figure 5 Recovering neurons (after cryopreservation with 10 wt% DMSO) observed by phase contrast microscopy.

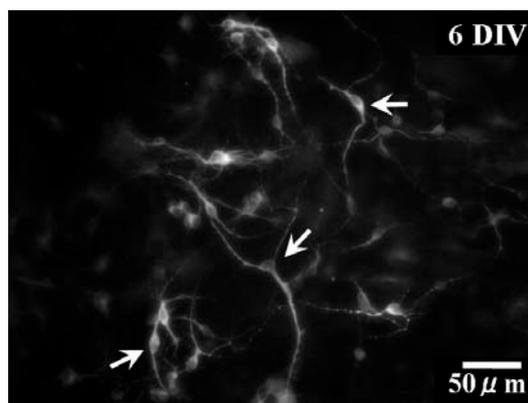


Figure 6 Cells stained with MAP2, a neuron-specific marker (after cryopreservation with 10 wt% DMSO).

The number of neurons recovered after treatment with 10 wt% DMSO was reduced to approximately 60% of that obtained after treatment with 5 wt% DMSO. Cells were not

present after cryopreservation with 20 wt% DMSO. These results indicate that neurons were successfully recovered after cryopreservation with low DMSO concentrations. Cryopreservation with 20 wt% DMSO appears to have a toxic effect on the cells.

The morphology of the cells was similar to that of the control neurons at DMSO concentrations of 5 wt% and 10 wt%. Compared to the control cells (as shown in Figure 1), it was observed that in the cryopreserved samples, glial cells abruptly regenerated a few days after thawing. It is suggested that these glial cells help neurons to grow after the latter recover from the cryopreservation conditions.

Furthermore, cryopreservation with 5 wt% DMSO preserves the nerve impulses of the recovering neurons as determined with the MED 64 system (Figure 7).

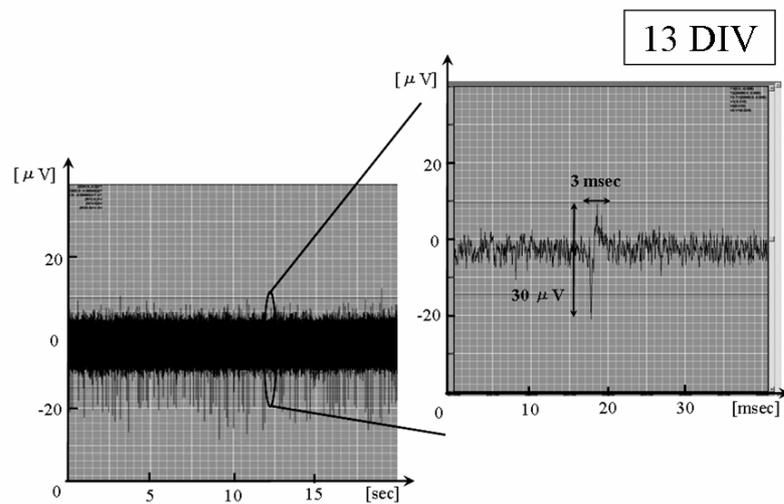


Figure 7 Nerve impulses of the recovering neurons (with 5 wt% DMSO) as determined with the MED 64 system.

As shown in the extended signal in Figure 3, the function of the neurons was assayed based on the presence of nerve impulses; these nerve impulses were equivalent to those of neurons that had not been cryopreserved (amplitude: $30 \mu\text{V}$, width of pulse: 3 ms). These results indicated that the DMSO-cryopreserved neurons (at DMSO concentrations of 5–10 wt%) can be recovered and cultured without any damage to their morphology or electrical activities.

When the cooling rate was varied, microscopic observations showed that the neurons recovered at slow cooling rates (10 K/min). However, at high cooling rates (10^2 K/min), the recovery of neurons was not observed. This result may be explained on the basis of the freezing process of the cell. The cryopreservation process of DMSO may be explained as follows: DMSO treatment may result in depression of the intracellular freezing point when the extracellular water begins to freeze.¹¹ After the cells become dehydrated, the intracellular fluid may be vitrified when the temperature approaches the glass-transition point. Thus, no intracellular ice crystals are formed, and DMSO protects the cells.¹²

3.3 Cryopreservation with trehalose

After cryopreservation with 17 wt% trehalose, neurons were assessed by microscopic observations and immunofluorescence staining using MAP2 (Figures 8,9).

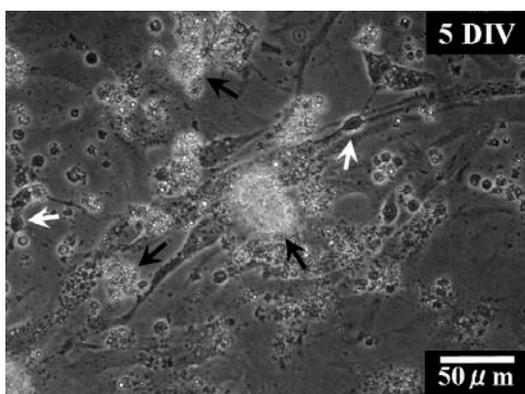


Figure 8 Recovering neurons (after cryopreservation with 17 wt% trehalose) examined by phase contrast microscopy.

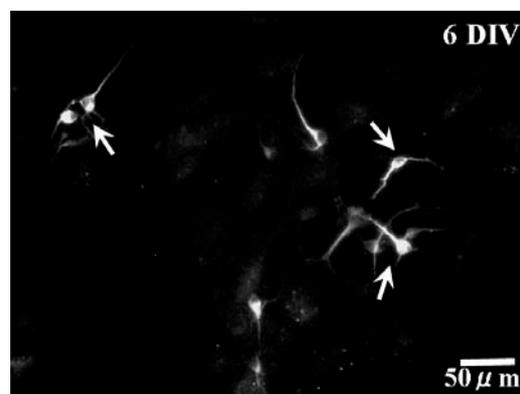


Figure 9 Cells stained with MAP2, a neuron-specific marker (after cryopreservation with 17 wt% trehalose).

Recovering neurons were not observed in the cryopreservation experiments with 3–10 wt% trehalose. Therefore, we consider that trehalose may only function as a cryoprotectant at concentrations of 17 wt% and possibly higher. In the cryopreservation experiments with 17 wt% trehalose, the recovery ratio of neurons was lower than that obtained with 5 wt% DMSO. The nerve impulses of the recovering neurons were not measured in this case because the recovery ratio of neurons was low. It has been reported that trehalose inhibits the growth of ice,¹³ and this property would prevent the disruption of the neuronal membrane.¹⁴ To determine if trehalose is a suitable cryoprotectant for neurons, further studies are necessary.

In comparison with trehalose alone, a mixture of trehalose and methanol has been reported to be useful for the cryopreservation of salmon sperm.¹⁵ However, using a mixture of 10 wt% trehalose and 8 wt% methanol, no significant improvement was observed in the cell morphology (Figure 10). In future studies, in order to determine the suitability of the mixture, we have to find additives that heighten the effect of trehalose.

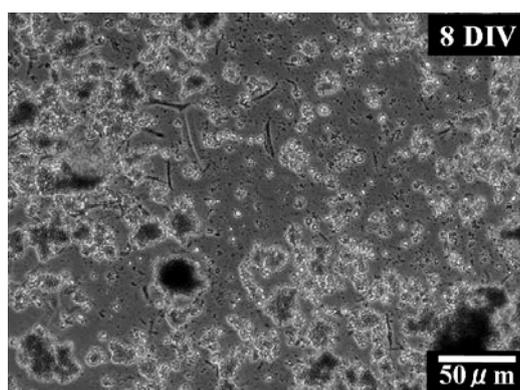


Figure 10 Recovering neurons (after cryopreservation with 10 wt% trehalose and 8 wt% methanol) examined by phase contrast microscopy.

4 CONCLUSIONS

We studied the cryopreservation of rat cortical cells using two effective cryoprotectants—DMSO and trehalose—at two different cooling rates. Microscopic observations and immunofluorescence staining with MAP2 revealed that the neurons had recovered after cryopreservation with 5–10 wt% DMSO. The recovering neurons showed nerve impulses that could be studied with the MED 64 system; these impulses were similar to those of neurons that had not been treated with cryopreservation. The neurons did not recover at high DMSO concentrations (20 wt%) because of the toxic effect of DMSO. For the cryopreservation of neurons, cooling in 5 wt% DMSO at 10 K/min was more suitable than at 10^2 K/min. Therefore, it appears that the appropriate conditions for the cryopreservation of cortical neurons are a DMSO concentration of approximately 5 wt% and a cooling rate of 10 K/min.

Some neurons recovered after cryopreservation in 17 wt% trehalose at a cooling rate of 10 K/min as determined by microscopic observations and immunofluorescence staining using MAP2; however, neurons did not recover at lower concentrations of trehalose. These results indicated that trehalose functions as a cryoprotectant only at high concentrations.

The difference in the recovery of cortical neurons in the presence of these additives might be a result of the cryopreservation process that is explained as follows: DMSO is taken up into the cell and allows vitrification of the intracellular solution, leading to the stabilization of the cell membrane. However, in the case of trehalose, the molecules may be too large to permeate into the cell; they may only contact the membrane extracellularly and protect the cell from freezing damage. To clarify the details of this cryoprotection mechanism, we need to study the process of cell freezing.

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