Cryopreservation of Brain Tissue for Primary Culture

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Abstract: Factors affecting recovery of brain cells from cryopreserved cerebral tissues of fetal rats were examined based on yields of viable cells on cell culture. Favorable preservation was obtained with freezing small pieces (less than 1 mm cube) of brain tissues rather than whole tissues or dissociated single cells, and use of 10% dimethylsulfoxide as a cryoprotectant in liquid nitrogen. As for cell preparation procedures, cell survival was improved when tissues were heated at 32°C during papain digestion and centrifugation. Under favorable conditions, the number of brain cells recovered from cryopreserved tissues corresponded to 20–30% of those from fresh control tissues. Immunocytochemical characteristics of cultured neurons, astrocytes, and oligodendrocytes from cryopreserved and fresh tissues were indistinguishable. Semi-quantitative analyses of microtubule-associated protein-2 (MAP-2) and synaptophysin revealed that there was no difference in the amounts of these markers between cultures from both fresh and cryopreserved tissues. These results suggest that most of all cell types including neurons were equally susceptible to the cryopreservation procedures. We concluded that cryopreservation in liquid nitrogen is an effective method for preservation of embryonic brain tissues for later use in cell culture studies.

Key words: brain, cerebral tissue, cryopreservation, primary culture, rat

Introduction

Cryopreservation of brain tissues is a traditional technique. There are many reports on cryopreservation of brain tissues of humans \([3, 5, 7, 10, 13, 15]\), primates \([2, 16]\), bovine \([8]\), and rats \([1, 4, 11, 12, 14, 17]\) for tissue transplantation or tissue culture. However, most of these reports focus on the ventral mesencephalon including dopamine neurons for transplantation against neurodegenerative diseases such as Parkinson’s disease \([1, 2, 5, 7, 12, 14, 16]\).

In this study, we tried to establish the favorable conditions and cell preparations that supported subsequent primary culture of cerebral neurons of fetal rats, which are most frequently used for primary culture of brain cells with detailed knowledge on their characteristics.

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To estimate the influence of cryopreservation on subsequent culture, immunocytochemical characteristics of three major cell types (neurons, astrocytes, and oligodendrocytes) in primary culture were compared between fresh and cryopreserved tissues. In addition, the amount of MAP-2, specific for neuronal cell bodies and dendrites, and synaptophysin, a membrane component of the synaptic vesicle, were also compared between fresh and cryopreserved tissues.

**Materials and Methods**

**Animals:** Pregnant Sprague-Dawley rats were purchased from CLEA Japan (Tokyo, Japan). The animals were maintained under controlled conditions (temperature, 24 ± 1°C; humidity, 55 ± 5%) in plastic cages with sterilized wood shavings for bedding and fed commercial diets, CMF (Oriental Yeast, Tokyo, Japan), with tap water ad libitum. This experiment was conducted according to the guidelines of the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

**Brain cell culture:** Brain cells were cultured according to the method described by Ichikawa et al. [6] with some modifications. Fetuses were removed on gestational day 18 under anesthesia with diethyl ether. Fetal brains were transferred into ice-cold isolation medium (IM) consisting of equal volumes of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) and Dulbecco’s Modified Eagle’s Medium with 1.2 mg/ml NaHCO₃, 110 μg/ml pyruvic acid, 25 μg/ml streptomycin, and 50 units/ml penicillin (mDMEM), and cut into cerebral hemispheres. After meninges were carefully removed, the cerebral hemispheres were rinsed and minced into small pieces less than 1 mm cube in culture medium (CM) (mDMEM with 5% fetal calf serum and 5% horse serum). Then tissue pieces were digested in PBS containing 1.5 U/ml papain, 0.1 mg/ml DNase, 0.2 mg/ml cysteine, 0.2 mg/ml albumin, and 5 mg/ml glucose at 32°C for 30 min. Cells were dissociated gently by several passages through a disposable pipette and centrifuged 3 times in CM at 1,200 rpm for 5 min at 32°C. Cells in CM were seeded onto a LAB-TEK chamber slide (Nalge Nunc, Tokyo, Japan) coated with 0.125% polyethyleneamine at 2.5 × 10⁵ cells/cm². All cultures were maintained at 37°C in humidified 95% air and 5% CO₂. A half volume of culture supernatant was replaced with pre-warmed CM once every week.

**Cryopreservation and thawing:** Brain tissues or cells were cryopreserved in culture medium supplemented with DMSO as cryoprotective medium. These samples were first frozen to -80°C in a freezing container (Cryo 1°C Freezing Container, Nalge Nunc, Tokyo, Japan) which provided a freezing rate of about -1°C/min, and then stored in liquid nitrogen for more than 1 week. Cryopreserved brain tissues or cells were thawed rapidly in a water bath at 32°C, and rinsed several times in IM to remove DMSO. Then, thawed brain tissues or cells were subsequently subjected to papain-digestion or centrifugation, respectively, and prepared as described above.

**Factors affecting recovery of viable brain cells after cryopreservation:** In order to determine the favorable conditions for cryopreservation and cell preparation that support subsequent primary culture of brain cells, the effects of the following factors on the recovery of viable cells were examined. The number of viable cells and the viability (viable cells / total cells × 100) were determined by trypan blue dye exclusion. **Size of tissue pieces:** Brain cells obtained by the method described above were cryopreserved under three different conditions: in the state of whole cerebral hemispheres, minced small pieces less than 1 mm cube, and papain-dissociated single cells. CM supplemented with 10% DMSO was used as cryoprotective medium. **Cryoprotectants:** Small pieces of brain were cryopreserved in CM supplemented with 0%, 5%, 10%, 15%, or 20% (v/v) DMSO. The results were compared in order to establish the most favorable cryoprotective medium. Subsequent procedures were the same as mentioned above. **Temperature during cell preparation:** Small tissue pieces cryopreserved in CM supplemented with 10% DMSO as cryoprotective medium were thawed and rinsed. After papain-digestion, cells were maintained ice-cold or at 32°C during centrifugation. Subsequent procedures were the same as mentioned above.

**Morphological studies:** Cells in culture were identified by immunostaining for cellular elements. Antibodies against microtubule associated protein-2 (MAP2) (1: 1,000, Boehringer Mannheim, Mannheim,
Germany) and neurofilament protein (NF) (1: 50, DAKO, Carpinteria, USA), glial fibrillary acidic protein (GFAP) (1: 1,000, DAKO), and myelin basic protein (MBP) (1: 250, Boehringer Mannheim) were used for identification of neurons, astrocytes and oligodendrocytes, respectively. After fixed with 2% paraformaldehyde and 8% sucrose in PBS for 1 h at 4°C, cultures were rinsed in PBS and incubated in a primary antibody diluted in PBS containing 2% (w/v) bovine serum albumin and 4% (v/v) horse serum for 30 min at 37°C. Cultures were subsequently rinsed in PBS and incubated in a biotinylated secondary antibody dilution. Peroxidase-labelled streptavidin (DAKO) and 3,3'-diaminobenzidine (WAKO Pure Chemicals, Tokyo, Japan) were used as enzyme reagent and substrate, respectively. Immunocytochemically stained cultures were observed without counterstaining.

Western blot analyses: For western blot analyses, cells were plated at 8.5 × 10^5 cells/cm^2. Total cellular protein was extracted by PBS containing 9.85 mg/ml Tris-HCl, 0.744 mg/ml ethylenediaminetetraacetic acid (EDTA), 0.348 mg/ml ammonium persulfate, 0.5% (v/v) TritonX-100, and 2.3% (w/v) SDS. Total protein was adjusted to 10 μg, and then developed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE with 12.5% acrylamide gel). The separated proteins in the gel were transferred and blotted onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA). After blocking with 5% nonfat-dried-milk in PBS and 0.1% Tween-20 for 1 h at room temperature, the blotted membranes were incubated in dilutions of primary antibodies against MAP-2 (1: 1,000, SIGMA, Saint Louis, USA) and synaptophysin (1: 1,000, DAKO) overnight at 4°C. Then they were incubated in horse-radish peroxidase conjugated secondary antibody dilution. The immunoreactions were visualized by enhanced chemiluminescence (ECL, Amersham, UK). Each band obtained was analyzed and quantified with software (Quantity One, PDI, Inc, NY, USA).

Statistical analysis: Statistical analysis was carried out with Student’s t-test.

**Results**

Factors affecting recovery of viable brain cells after cryopreservation

Size of tissue pieces: Recovery of viable cells was significantly higher when brain cells were cryopreserved in the form of small pieces than whole cerebral hemispheres (Fig. 1A) or single cells dissociated by papain treatment before cryopreservation (Fig. 1B). Viability of the cells recovered from single cells was remarkably low (8.2%) compared with that from small pieces (88.0%) and whole cerebral hemispheres (88.5%).

Cryoprotectants: Among the concentrations of DMSO tested, 10% (v/v) gave the highest recovery of viable cells after cryopreservation (Fig. 1C). Viability of the cells recovered from 10% DMSO cryopreservation was 90.2%. DMSO at 5% and 15% gave moderate recovery of viable cells (Fig. 1C) and viability (63.3% and 70.5%, respectively). Few cells survived when they
were cryopreserved in 0% and 20% DMSO (Fig. 1C), with viabilities of 30.6% and 9.2%, respectively. A commercial cryoprotectant was less effective than 10% DMSO (data not shown).

Temperature during cell preparation: The number of viable cells was significantly higher when cells were maintained at 32°C during the purification procedure than at the ice-cold temperature (Fig. 1D). Viability was also higher when the cells were purified at 32°C (89.0%) than at ice-cold (78.3%). No aggregation of cells was observed during preparation at 32°C.

Favorable procedure: In the present evaluation, the most favorable procedure for cryopreservation and cell culture was when brain cells were cryopreserved as minced small tissue pieces in CM supplemented with 10% DMSO, and thawed cells were maintained at 32°C during preparation after papain-digestion. When the reduction of viable cells by cryopreservation using the most favorable procedure was evaluated through 8 experiments (twice/dam) with cryopreservation and 4 experiments (once/dam) without cryopreservation from additional 4 dams, 3.58 ± 0.45 × 10⁶ viable cells/cerebral hemisphere (mean ± SD) with a viability of 89.0% were recovered after cryopreservation. This number was 20–30% of that obtained from fresh tissues (13.65 ± 2.98 × 10⁶ viable cells/cerebral hemisphere) with a viability of 84.5%. The number of viable cells after centrifugation was around 75% of that before the centrifugation, and cell viability was slightly increased (data not shown), while the debris of tissues were removed by centrifugation.

Morphological studies

In these experiments, equal numbers of cells prepared from the cryopreserved tissues by the most favorable procedure described above and those from the fresh control tissues were seeded on a plate and their characteristics in culture were compared. Neurons cultured from the cryopreserved tissues began extending a few dendrites at 1 day in culture (data not shown) and they had many dendrites at 7 days in culture (Fig. 2A). At 14 days in culture, complicated
networks of dendrites were observed (Fig. 2B). Neurons cultured from the fresh tissues showed characteristics indistinguishable from those of cryopreserved tissues (Fig. 2, C and D). GFAP positive cells (astrocytes) made a monolayer at 7 days in culture (Fig. 3, A and D) in cultures from both the cryopreserved and fresh tissues. A few MBP positive cells (oligodendrocytes) were first observed at 3 days in culture (Fig. 3, B and E) and the number of MBP positive cells increased until 21 days in culture in cultures of both the cryopreserved and fresh tissues. The density of NF positive cells, which showed much differentiated morphology with long dendrites, was very low at 14 days in culture in cultures of the cryopreserved and fresh tissues (Fig. 3, C and F).

Quantitative comparison of specific proteins in primary culture: The results of semi-quantification of MAP-2 and synaptophysin by western blot analysis revealed that the amount of each protein was not different in cultures of the cryopreserved and fresh tissues (Fig. 4, A and B) (p>0.5). Although the concentration of total protein calculated from protein (μg) per 1 mm² of the cryopreserved tissue (0.481 ± 0.022 μg/mm², mean ± SEM) was significantly lower than that of the fresh
tissue (0.575 ± 0.020 µg/mm²) at 1 day in culture (p<0.01), this value showed no difference between cultures from the cryopreserved and fresh tissues at 7 days in culture.

Discussion

In this study, we established a favorable procedure for the successful cryopreservation and primary culture of brain cells of fetal rats. Usually, fetuses on gestational day 16–18 are used for primary culture of rat neurons, since younger brains yield few cells and neurons from later developmental stages are vulnerable to cell isolation procedures. Under the culture conditions employed in the present study, a primary culture of 18-day fetal brains resulted in a complicated network of neurons with astrocytes and oligodendrocytes within two weeks. This mixed culture system may represent well the in vivo neural network.

The recovery of viable cells from the tissues cryopreserved in the form of whole cerebral hemispheres was somewhat low compared with that from the tissues cryopreserved as small pieces. This can be explained by the differences in the time for DMSO to permeate into the tissues; a whole cerebral hemisphere was too large for DMSO to penetrate into all the tissue before reaching the freezing temperature. On the other hand, the remarkably low recovery and viability from cryopreserved single cells could be the result of damage to each single cell during the papain-dissociation. It is probable that direct freezing deprived single cells of enough time to recover from the membrane damage caused by the enzyme. In contrast to the present findings, cryopreservation of dissociated single neurons of humans was relatively successful in a previous study [10], although the conditions of freezing and thawing were basically the same. However, it was also reported that the efficacy of the cryopreservation procedure was dependent upon the gestational age of the brain tissue. We think that this discrepancy is due to the difference in the developmental stage and the species, and there might be a more appropriate gestational day for cryopreservation of rat cerebral cells in the state of dissociated single cells.

The highest recovery of viable cells was obtained using 10% DMSO as a cryoprotectant, which is in accordance with previously reported results of 1 M (7.8%) [4], and 7.5–10% [15] as favorable DMSO concentrations. In many other studies, 7–10% DMSO was used as cryoprotectant. There is probably a favorable concentration of DMSO between 7–10% for each type of cell from each of the brain regions, species, and stages of differentiation.

The improvement of the recovery of viable cells by maintaining at 32°C during cell preparation suggests that it is ineffective to work on ice after papain digestion at 32°C, and that brain cells are highly sensitive to changes of temperature. It is generally accepted that cells should be maintained at an ice-cold temperature before seeding on the plate. The idea is based on the concept that cells with a low metabolic level are resistant to physical and enzymatic damage. The present result suggests that temperature change may also be harmful for brain cells, and that suitable environmental conditions should be defined for each cell type and circumstance. No negative effect, or aggregation of cells, was found at 32°C in this study.

The morphological studies revealed that the cryopreservation did not influence extension of dendrites of neurons, glial cell growth and differentiation of oligodendrocytes in culture. From these findings, we concluded that the cryopreservation procedure did not selectively injure particular cell types among neurons, astrocytes, and oligodendrocytes. The present results are in agreement with those described before [1], that there is no morphological difference in cultured mesencephalic neurons from fresh and cryopreserved tissues of fetal rats. However, whether cryopreservation has any influence on physiological and functional characteristics remains to be investigated.
The low density of protein of whole cultured cells from cryopreserved tissue at 1 day in culture is thought to be the consequence of the reducing number of cells attached to culture plates. This finding is in agreement with a previous description of rat hippocampal cells [17], in which cryopreservation had a significant influence on the viability after dissociation and survival in culture. However, this negative effect was not observed at 7 days in culture in this study. There were no differences in the amounts of MAP-2 and synaptophysin between primary cultures both from the fresh and cryopreserved tissues at 7 days in culture. These findings, which were in accordance with the morphological study, suggest that cryopreservation does not influence neuronal differentiation and synapse formation in culture.

The recovery of viable cells from the cryopreserved tissues was 20–30% of the number recovered from the fresh tissues. This result was not so excellent when compared with those appearing in the literature, although direct comparison with other reports is impossible because of differences in species, collected brain regions, developmental stages, and definitions of viable cells. To improve the recovery rate of viable cells and viability, further investigations into the most favorable conditions for cryopreservation and cell preparation procedure are required.

The method of cryopreservation described here could be applied, with appropriate adjustment, to the primary culture of brain cells of any animal species. The protocol described in the present study would provide successful cryopreservation of fetal brain cells of humans, non-human primates and other uncommon animals at a rare opportunity of collection, and enable us to store and distribute these tissues, prior to culture for extended, prolonged, and well-designed studies. In addition, cryopreservation of these precious tissues would also lead to a reduction in the number of mothers required for primary culture, because appropriately aliquoted fetal tissues from one mother can provide many opportunities for primary culture.

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References