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Freezing behavior of adherent neuron-like cells and morphological change and viability of post-thaw cells ☆

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ABSTRACT

Freezing of nerve cells forming a neuronal network has largely been neglected, despite the fact that the cryopreservation of nerve cells benefits the study of cells in the areas of medicine and poison screening. Freezing of nerve cells is also attractive for studying cell morphology because of the characteristic long, thread-like neurites extending from the cell body. In the present study, freezing of neuron-like cells adhering to the substrate (differentiated PC12 cells), in physiological saline, was investigated in order to understand the fundamental freezing and thawing characteristics of nerve cells with neurites. The microscopic freezing behavior of cells under different cooling rates was observed. Next, the post-thaw morphological changes in the cells, including the cytoskeleton, were investigated and post-thaw cell viability was evaluated by dye exclusion using propidium iodide. Two categories of morphological changes, beading and shortening of the neurites, were found and quantified. Also, the morphological changes of neurites due to osmotic stress from sodium chloride were studied to gain a better understanding of causation. The results showed that morphological changes and cell death were promoted with a decrease in end temperature during freezing.

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Introduction

Cryopreservation has been used for the long-term storage of cells such as blood cells, spermatozoa and embryos, tissues such as skin and bone marrow, and foods in a wide range of fields including medical care and agriculture [3,7]. The post-thaw viability of biological materials (cells and tissues) is an important index for assessing the quality of cryopreserved materials. Viability is usually defined as a specific function-retention rate of the materials compared to their intact form, and it depends on the type of material, the type and concentration of cryoprotectants, and the thermal history during cooling and warming [29,48]. The thermally controllable factors, including cooling rate, warming rate and storage temperature, affect the state of the unfrozen solution, ice crystals and ice–cell interaction at the cellular scale, and the structure of water, ice crystals and biopolymers at the molecular scale, and thus determine the viability of the material. However, the mechanisms of freezing injury and protection by cryoprotectants are not well understood. A better understanding of the relationship between microscopic freezing and thawing behavior and the post-thaw viability of various types of materials is essential [14,15], and has practical benefits for extending the coverage of cryopreservation to different material types and thermal conditions.

The freezing of suspended cells without neurites, including immature cells and mature cells that have lost their neurites due to suspension, has mainly been studied [8,17,18,32,36,45] for the application of cryopreservation to the efficient preparation of cells for neuronal cell transplantation and neuropharmacological drug screening. In drug screening, fresh neurons, cryopreserved neurons and sliced fresh neural tissues have been used. However, a laborious procedure for inducing differentiation so that the cells extend their neurites and build a neuronal network is usually needed prior to screening when neuronal cells in suspension without neurites are used, whether fresh or cryopreserved cells. The cryopreservation of nerve cells, including their neuronal network formed *in vitro*, could contribute to increasing the efficiency of screening using nerve cells. For screening using cryopreserved sliced neural tissues, an *in vivo* neuronal network needs to be preserved in the tissues.

Some work has been done on the cryopreservation of neuronal networks. For example, Ma et al. [23] tried to cryopreserve primary neurons forming a network *in vitro*. They demonstrated that the

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method of trapping cells in a collagen gel during cryopreservation is effective for improving post-thaw viability, as shown by the normality of dye exclusion, intracellular enzymatic activity, and recycling of the synaptic vesicles. Similar work with alginate gel has also demonstrated its effectiveness for improving the intracellular enzymatic activity of cryopreserved neuron-like cells [24]. Pichugin et al. [33] attempted to vitrify hippocampus slices 475 μm thick, and evaluated post-thaw viability based on the ratio of $[K^+]/[Na^+]$ in the slice. They showed that viability was improved compared to ordinary preservation by freezing (viability reached more than 90% of that of the unfrozen control). However, many aspects of cryopreservation remain unclear. For example, neurotransmission of cryopreserved neurons has not been well investigated electrophysiologically. Also, fundamental characteristics, including the freezing and thawing behavior of cells and the dependence of post-thaw viability on the cooling conditions, have not been investigated in detail.

The present study aims to understand the freezing and thawing of nerve cells with neurites that are constituents of a neuronal network. The freezing of neuron-like cells with neurites adhering to the substrate in physiological saline was investigated. Neurons with neurites have an important application as experimental materials in the pharmacological industry, and are also attractive for studying the influence of cell morphology on freezing because of their characteristic long, thread-like neurites extending from the cell body. Furthermore, this study could benefit cryotherapy such as cryosurgery, which occasionally involves complications such as temporary disruption of neurotransmission, which can last from several hours to weeks [9,13].

Microscopic freezing behavior, such as freezing patterns, the morphology of ice crystals and interactions between ice crystals and cells, was observed under different cooling conditions. Next, morphological changes of the cells after freezing and thawing were observed in order to quantify typical morphological changes: the beading and shortening of neurites. Also, the cytoskeleton (intermediate filament) was visualized by immunocytochemistry and investigated. In particular, the influence of osmotic stress on morphological changes was investigated. Finally, post-thaw cell viability was evaluated by dye exclusion using propidium iodide. The relationships between the above characteristics are discussed.

Materials and methods

Preparation of adherent neuron-like cells

A PC12 cell line (Riken Cell Bank, Japan) derived from rat adrenal pheochromocytoma was used, because this cell line is commonly used as a model nerve cell [11,46].

The PC12 cells were maintained on a plastic culture dish (ϕ 50 mm, BD Biosciences, USA) at 37 °C in air with 10% CO_2. The medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies Corp., USA), 5.0 v/v% fetal bovine serum (FBS) (Equitech Bio, Inc., USA), and 5.0 v/v% horse serum (HS) (Life Technologies Corp., USA), and this was subcultured every 6–8 days.

The method used to induce neuronal differentiation of PC12 cells is summarized as follows.

(1) Two kinds of substrates were used to prepare adhered neurons with neurites, depending on the two kinds of cryostages used for freezing a sample under the microscope, as described below. (a) One was a plastic chamber slide (polymethylpentene, 4 wells, 8.9 × 20.2 mm/well, base 1.0 mm thick, Lab-tek chamber slide system, Thermo Fisher Scientific Inc., USA) for a directional solidification stage. The chamber slide consisted of a chamber wall and a microslide. (b) The other was a plastic coverslip (polyphenylene sulfide, ϕ13 mm, base 170–200 μm thick, Thermanox coverslips, Thermo Fisher Scientific Inc., USA) placed in a plastic culture dish (ϕ35 mm, BD Biosciences, USA) for a cryostage of uniform temperature distribution. The chamber slide in (a) and the coverslip placed in the dish in (b) were coated with collagen (Type I collagen from calf skin, Elastin Products Co., Inc., USA).

(2) The cells were seeded, at a cell density of around 1500 cells/cm^2 in a fresh medium used for the passage culture, in the chamber slide or the dish with the coverslip prepared in (1), and incubated at 37 °C under 10% CO_2.

(3) The medium used in (2) was changed to DMEM containing 100 ng/ml NGF (NGF-7S from murine submaxillary gland, Sigma–Aldrich Co. LLC, USA), 0.05 v/v% FBS and 0.05 v/v% HS 24 h after the seeding, and then the medium was changed to a fresh one every 2 days while the cells were incubated. Within 24 h of the exposure of cells to NGF, the cells began to differentiate: cells with a flat cell body and long neurites emerged. The number and number fraction of differentiated cells increased with the passage of time.

(4) In this study, the cells with neurites longer than the diameter of the cell body were defined as differentiated cells [6,44]. When the cells on the substrates attained a number fraction of such differentiated cells larger than 80%, the cells were used as a sample for experiments on freezing. The percentage was achieved between 6 and 10 days after the exposure of cells to NGF.

Cryostages for freezing and thawing

Two kind of cryostages attached to a microscope – a directional solidification stage (DSS) [15,35] and a uniform temperature distribution stage (UTDS) (10002L, Linkam Scientific Instruments Ltd., UK) [22,47] – were used for the freezing and thawing of samples.

The DSS consists of two copper blocks, one with a higher temperature and one with a lower temperature than the freezing temperature of the sample, which are separated by a gap of 2 mm. If the sample on the stage is moved at an advancing velocity of V mm/min under the temperature gradient G °C/mm across the gap from the higher-temperature block to the lower-temperature block, then the sample is cooled at a cooling rate of $H = G{\cdot}V$ °C/min. If the sample is moved in the opposite direction, it is warmed at a warming rate of $W = G{\cdot}V$ °C/min. The DSS has some advantages and disadvantages compared to the UTDS. The advantages are that continuous movement of the sample needs no ice seeding to start the freezing of a sample and allows more cells to be focused on and observed during the freezing of one sample. The disadvantages are that it takes longer for the freezing and thawing of a sample, particularly in the range of the slow cooling rate, and the region of fixed spot observation is limited temporally and spatially.

The UTDS consists of a silver cylindrical block with an open hole (ϕ2 mm) at the center for the light path. The sample on the stage has a uniform temperature distribution, which allows fixed-spot observation during freezing and thawing. In the present study, ice seeding of the sample was performed at −1.0 °C by bringing the tip of a stainless-steel spatula 0.85 mm thick cooled in liquid nitrogen into contact with the edge of the upper glass coverslip of the sample.

For both stages, a very small amount of ethanol was injected into a thin gap between the lower surface of the substrate of the sample and the upper surface of the stage, thereby achieving a good thermal contact. The cooling rate and warming rate of the sample were determined by measuring the temperature of the sample in time series with chromel–alumel thermocouples (ϕ 0.1 mm and ϕ0.05 mm) and a data logger (Thermodac-F, Eto Denki Co., Japan).

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