Development of Pathology Specimen Preparation Method by Supercooling Cryopreservation under Magnetic Field

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Abstract

Brain tumors have the highest incidence of childhood solid cancers and they also have the highest mortality rate among childhood cancers. One factor cited for improved prognosis in cancer is improvement in surgical extraction rate, but types of childhood brain tumors are more diverse in comparison to those of adults, and it is at present exceedingly difficult for even an experienced pathologist to perform an accurate diagnosis. We have developed a technique for freezing under magnetic field for the purpose of internal organ cryopreservation, and we conducted this study after considering that this freezing technique could be useful for rapid diagnosis utilizing frozen tissue during surgery. Results showed the arrangement of neurons to be in much better order with brain tissue frozen under magnetic field than that which was frozen by liquid nitrogen. For pancreatic tissue, it was found that insulin staining was clearer for freezing performed under magnetic field than with liquid nitrogen. In short, we found that this technique is not simply for the preservation of tissue, but has the potential to improve the accuracy of pathological diagnosis and surgical extraction rate as well as limiting chemotherapy and radiation therapy. This is the principal outcome that will contribute to an improved quality of life (QOL) for childhood cancer patients.

Keywords

Technique of freezing under magnetic field, Supercooling freezing, Brain tumor, QOL of childhood, Pathological diagnosis

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Introduction

Childhood brain tumors

According to the U.S. National Cancer Institute (NCI) 2006 statistics, the incidence of brain tumor in children under 15 was 3.2 per 100,000 children (SEER Program 1975-2006), and brain tumors have the highest incidence of childhood solid cancers. With the progress of medical treatments, the 5-year survival rate for childhood leukemia has surpassed 70%. Meanwhile, the cancer mortality rate for those under 20 is highest for brain tumors.

It is now possible to safely and accurately diagnose most brain tumors with CT or MRI scans, however, improvement in surgical extraction rate is required to further improve prognosis. Improvement is needed not only in surgical technique, but in accuracy of pathological diagnosis as well. However, this is a difficult task because of no clear boundary between normal and cancerous tissue. Furthermore, it is extremely difficult for even an experienced pathologist to make an accurate diagnosis since: factors such as the site of occurrence and the type of tumor differ between childhood and adult brain tumors; the malignancy of the tumor may vary with location even within the same tissue type; and tumor types are extremely diverse, numbering more than 100.

The procedure, termed, intra-operative rapid diagnosis, in which the pathologist examines under microscopy tumor tissue collected from the patient and makes a diagnosis, is for purposes of determining the tissue type of tumor and its malignancy as well as the extent of extraction. This is an important diagnostic technique influencing the surgical success rate. Since the cryosection is not good for the preservation of cell imagery, it may be necessary to wait for a permanent preparation (paraffin section), which takes 2-3 days. The history of permanent preparation dates back 100 years. While there have been improvements in the manufacturing techniques of specimens and reagents, there has been no advancement in the cryosection manufacturing method, especially in the freezing process.

Late effects

Chemotherapy or radiation therapy is employed in the treatment of brain tumors when the tumor cannot be excised through surgery, or when the tumor occurs in a site where surgery would be too difficult. In these cases, side effects (late effects) of these cancer therapies remain after treatment. There is an especially high possibility of inducing serious developmental disorders of the central nervous system when radiation therapy is used on children under 3 years old; with damage to central nervous system, pituitary gland, and hypothalamus, various disorders including short stature, anorexia, obesity, paralysis, dyskinesia, spasm, speech disorders, loss of vision, impaired intellect, and mental defects occur (Figure 1). Among brain tumors, it is reported a 5-year survival rate for osteoblastoma was over 80%. And so, hereafter, treatment strategies, which not only improve survival rate, but also improve QOL following cancer treatment will be required.

Figure 1.

A 1-year and 3-month-old boy with rhabdoid tumor
Remaining tumor resection and free rectus abdominis musculocutaneous flap transplantation was performed after high-dose chemotherapy. It must be needed to diagnose tumor invasion into the eyeball, optic nerve or olfactory nerve during surgery because of skull base tumors. As a consequence of diagnosis, Eye enucleation was performed since tumor invasion into the tissue surrounding the eyeball was realized. Resection of olfactory nerve without tumor invasion was unnecessary.

Organ freezing techniques

With the preservation of fertility in child and young women cancer patient’s as our goal, we integrated super-microsurgery techniques from the medical field, freezing system developments from engineering, and food cryopreservation techniques of the food industry, to develop a technique for the freezing of organs which was hitherto deemed impossible. We propose a new cryosection production method utilizing this special freezing technique based on supercooling under a variable
magnetic field applied to the organ.

Method

Freezing of mouse brain experiment

A male mouse (30 g) was euthanized under etherization and the brain was extracted. A 5 mm square of tissue was cut out and embedded in OCT and frozen as follows with liquid nitrogen or a freezing system producing a variable magnetic field (hereinafter referred to as magnetic field freezing system, AB1).

The brine tank of the freezing system was filled with 60% ethylene glycol, the samples was enclosed in a nylon pack from which air was expelled to the extent possible, the nylon pack were inserted into a weighted gauge and immersed in the brine layer. Magnetic field intensity was set to 0.1-0.2 mT, and the specimen was frozen at -40°C. After freezing, the specimen was stored until the experiment in a -80°C freezer.

Rat brain/pancreas freezing experiment

A female Lewis rat (300 g) was euthanized under etherization by blood removal, and the brain and pancreas were removed.

The cerebrum and cerebellum were removed, and after a transection along the midline, these were immersed in tubes filled with 1.5 ml saline solution. The left cerebral hemisphere and left cerebellum were frozen with liquid nitrogen as controls. The right cerebral hemisphere and right cerebellum were frozen with the magnetic field freezing system. Squares of 3-5 mm were cut from the pancreas and frozen in the same manner using either liquid nitrogen or the magnetic field freezing system. After freezing, the specimens were stored until the experiment in a -80°C freezer.

Histological and immunohistochemical examination

A cryosection 6 mm in thickness was prepared with conventional methods with the frozen mouse brain specimen embedded in OCT compound, HE staining was performed, and it was examined under microscopy.

The frozen rat brain specimen was thawed at 37°C for 5 minutes, after fixation in 10% formalin, it was embedded in paraffin, and thereafter a 4 mm section was prepared.

After deparaffinization, activation was performed for 20 minutes in a 10 mM citric acid buffer solution (pH6.0) at 98°C. After processing with 3% hydrogen peroxide, it was washed with phosphate buffered saline (PBS), and was allowed to react with the primary antibody, mouse anti-neurofilament NF-L monoclonal antibody (clone 2F11, 1:800, Dako), for 30 minutes at room temperature. After washing with PBS, it was allowed to react with Simple Stain Rat MAX-PO (MULTI) (Nichirei Bioscience) as a secondary antibody for 30 minutes at room temperature. After washing with PBS and DAB color development processing, nuclear staining was performed with Mayer’s Hematoxylin Protocol and the section was mounted.

The pancreas paraffin section was deparaffinized, processed with 3% hydrogen peroxide, then washed with PBS, and allowed to react with the primary antibodies, marmot antiinsulin antibody (1:400, Dako) and rabbit anti-glucagon antibody (previously diluted, Dako) for 30 minutes at room temperature. After washing with PBS, it was allowed to react with Simple Stain Rat MAX-PO (MULTI) as well as with Simple Stain Mouse MAX-PO (R) (Nichirei Bioscience) as secondary antibodies for 30 minutes at room temperature. After washing with PBS and DAB color development processing, nuclear staining was performed with Mayer’s Hematoxylin Protocol and the section was mounted.

Results

The HE stained brain tissue was assessed by means of microscopic examination, and it was found that the nuclei were clearer in the sample frozen with the magnetic field freezing system than the one frozen with liquid nitrogen, and the overall structure was also better maintained (Figure 2).

Intermediate filaments, one of the filaments that compose the cytoskeleton, exist in three varieties, NF-L, NF-M, and NFH. It is known that these appear in normal nerve cells, neuroendocrine cells, or some types of brain tumors. NF-L was employed as a neuronal marker to assess brain tissue structure and staining after freezing. The results showed no difference in staining dynamics with freezing technique, but specimens frozen with the magnetic field
freezing system showed better order of neuronal arrangement in both the cerebrum and cerebellum, the so-called "neural network" was preserved (Figure 3). Meanwhile, some of this network structure was destroyed in the specimen frozen with liquid nitrogen (Figure 3). Insulin and glucagon staining was performed on the pancreas to assess the structure of islets of Langerhans after freezing (Figure 4). Results showed, especially with insulin staining, 20% stronger staining for the specimen frozen with magnetic field freezing than the one frozen with liquid nitrogen.

Discussion
While pathological diagnosis from pathology specimen imagery plays an essential role in determining the character of tumor and the extent of surgical extraction, there has been nearly no advancement in the freezing method for rapid intra-operative pathological diagnosis. Accordingly, we have frozen brain and pancreatic specimens utilizing the magnetic field freezing system we have developed, and shown the usefulness of this system in the preparation of these pathological specimens.

Rapid freezing method using liquid nitrogen and slow freezing method using a programmable freezer have been identified as methods of freezing tissue and organs. We have developed a new freezing technique of freezing under variable magnetic field, which we call "supercooling cryopreservation" or "magnetic resonance influence (MRI) freezing". We have considered that tissue destruction in organ freezing might be controlled by inhibiting ice crystal growth and osmotic pressure changes through maintenance of supercooling under a variable magnetic field. As evidence, we have demonstrated that supercooling can be stably maintained at a more reduced temperature with the application of a variable magnetic field within a defined frequency range. By inducing supercooling at lower temperatures we inhibit cellular membrane disruption and the outflow of intracellular proteins.

The magnetic freezing system employed in this study produced magnetic fields with a broad frequency component (Figure 5). However, the results of this study and the work of Niino et al. strongly suggest the possibility that the optimal variable magnetic field frequency and intensity for freezing varies according to the tissue or cells, and more detailed examination here is needed.

Conclusion
With this study we wanted to put special focus on the freezing technique itself. The main items examined up to now in cryosection preparation include the microscope slide, fixation fluid, segment thickness, antibodies, and washing. So, we proposed an innovative pathology specimen production method from the completely new viewpoint of tissue freezing under variable magnetic field. This technique has
Figure 3. NF-L immunohistochemical staining

Paraffin sections were produced after thawing of rat brain tissue frozen with liquid nitrogen or under magnetic field, and NF-L immunohistochemical staining was performed.

a. NF-L stained cerebrum. Liquid nitrogen (left). Freezing under magnetic field (right).

b. NF-L stained cerebellum. Liquid nitrogen (left). Freezing under magnetic field (right).

Figure 4. Insulin/glucagon immunohistochemical staining of pancreas

Paraffin sections were produced after thawing of rat pancreas tissue frozen with liquid nitrogen or under magnetic field, and insulin and glucagon immunohistochemical staining was performed.

a. Insulin stained islets of Langerhans shown. Liquid nitrogen (left). Freezing under magnetic field (right).

b. Glucagon stained islets of Langerhans shown. Liquid nitrogen (left). Freezing under magnetic field (right).
the potential to increase the precision of pathological diagnosis and surgical extraction, as well as limiting the use of chemotherapy and radiation therapy. Moreover, it is an important result in that it contributes to QOL improvement in childhood cancer patients.

**Issue 1: Differences in freezing requirements for each tissue**
We assume that there is an optimum magnetic field intensity, frequency, and freezing point for each tissue. Investigation of these requirements is our challenge hereafter.

**Issue 2: Improvement in freezing speed**
For use in intra-operative, rapid diagnosis, tissue must be frozen in 15-30 seconds, within one minute at the longest. Considering portability, energy conservation, safety, and magnetic shielding, the development of a magnetic field freezing system with a high freezing performance is essential.

Figure 5. Magnetic field frequency measurement in magnetic field freezing system
Measurements of magnetic field frequency of the magnetic field freezing system in this study were made with a magnetic field measuring instrument that is also employed in food cryopreservation. A probe was inserted into the brine tank of a magnetic field freezing system with an approximately 1 mT variable magnetic field induced, resulting values are shown. A frequency peak was found at approximately 250 kHz, and this frequency component was verified around that point, showing that the freezing system was producing a variable magnetic field with a broad frequency component.

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