Collaboration Super-Microsurgery * Food freezing technology

MRI, Magnetic Resonance Influenced, organ freezing method under magnetic field

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Abstract

A scientific approach towards organ preservation was initiated by Alexis Carrel, who was awarded the Nobel Prize in 1904. Subsequently, cryopreservation of organs gained momentum in the 1950s. However, organ cryopreservation has long been considered impossible, because the cells in the organs suffer from substantial damage due to ice-crystal formation and generation of osmotic-pressure gradients in the tissue. Meanwhile, in gynecology, freezing techniques for sperms, ova, fertilized eggs, etc., have been developed to ensure retention of fertility. Cells and tissue fragments can be frozen with a high probability of success. Some studies on the clinical applications of these approaches in humans have been published. In this study, we tried to cryopreserve organs, and not the cells or tissues, by using a freezing technology that employed a variable magnetic field. This freezing technology was principally developed by a Japanese company engaged in the development of food-freezing technologies. The combination of different techniques, i.e., the integration of this freezing technology with our technique, Super-Microsurgery, is expected to expedite basic experimental research on cryopreservation of small organs, particularly reproductive organs, which was hitherto considered impossible.

Keyword Super-Microsurgery, Ovarian transplantation, Testicular transplantation, Organ cryopreservation

Introduction

Preservation of organs

The Nobel laureate Alexis Carrel, who was a pioneer in the field of organ transplant and preservation, initiated trials on organ preservation, tissue culture, and artificial heart-lung machines. In organ preservation, it is important to preserve the organs for a long time without reducing the survival rate of the cells. To this end, studies are being conducted to develop approaches to avoid the various organ injuries that are associated with extracorporeal preservation and transplantation: e.g., warm ischemic disorder due to interruption of the bloodstream prior to the removal of the organ from the body, damage during preservation, and injury during bloodstream resumption (reperfusion).

Various temperatures, including normal temperature, refrigeration, supercooling, and freezing, have been used for organ preservation. The results showed that preservation by refrigeration (0–4°C) was the most effective method for lowering the metabolism to an optimum level and avoiding anoxic injury¹⁾. Studies have also focused on supercooling temperatures (–2°C to –8°C); at these temperatures, the organs are not frozen, and the metabolic levels can be lowered further while avoiding the cellular damage caused by freezing. However, this approach has not yet been applied for clinical purposes²⁾.

Currently, the most reliable and widespread organ-preservation method is the simple immersion method that is

performed using the University of Wisconsin solution—an organ-storage solution^{3, 4)}. However, there is a limit to the duration of storage in this method: 3 days for kidney, 20 hours for liver and pancreas, 4–6 hours for heart, and 8–12 hours for lungs. This duration of storage is not sufficient for pretransplantation preservation. While various freezing-preservation techniques have been tried to achieve longer storage durations, cryopreservation is still considered impossible. The specific issues associated with cryopreservation of cells/tissues are ice-crystal formation and hypertonic damage related to the concentration of the intercellular fluid. Since it is difficult to freeze all the cells of a large-volume organ under a given condition, these problems are particularly complicated. Further, since the component cells of a parenchymal organ are not identical, the optimal temperature may differ from cell to cell. The freezing temperature/speed and the protective solution may have to be selected, and the appropriate freezing conditions may have to be determined for each organ.

Potential of using super-microsurgery for the transplantation of small organs

Vascular anastomotic technology is indispensable for organ transplantation. Alexis Carrel applied this technology and successfully conducted autologous heterotopic transplantation of sheep kidney. In those days, vascular anastomosis was considered to be impossible. The development of vascular anastomotic technology actually led to progress in transplantation. From this perspective, we believe that our

super-microsurgical technology will also benefit basic research areas. Using super-microsurgery, the vascular anastomosis can be applied to up to 0.3–0.5 mm thick vessels⁵⁾, and therefore, a new experimental transplant model can be developed in small animals like mice and rats^{6,7)}.

Freezing under a variable magnetic field

A Japanese company engaged in the development of food-freezing technologies developed a technology for freezing food

without compromising on the quality of the thawed food. In this technology, food is frozen using a variable magnetic field that causes minimal damage to the cells (Figure. 4). We think that an organ-preservation technique based on this cryopreservation technology will reduce cell injuries and enhance the survivability of the preserved cells.

Necessity and urgency of cryopreservation of reproductive organs

Chemotherapy and radiotherapy are indispensable for

Water molecules in the organ prior to freezing

Freezing procedure

When the organ is exposed to a combination of multiple ultraweak energies, water molecules in the organ are vibrated, ice crystallization of the water content is inhibited, and the supercooled state is maintained.

Frozen state

The entire organ in the supercooled state is instantaneously frozen.

Post-thaw state

Since the pre-thaw state of water molecules is maintained even after thawing, the breakdown of the cells in the organ is minimized.

Freezing in a variable magnetic field

Conventional thawing technology

When the organ is directly exposed to cold air (−40°C to −50°C), ice crystallization is initiated, and the ice on the surface obstructs the freezing of the internal layers, thereby causing multilayered freezing.

The ice on the surface grows until the entire organ has frozen. The water molecules in the internal unfrozen segment are mobilized and sucked up toward the core of ice on the surface by capillary phenomenon. Then, this water evaporates, causing loss of water content and dryness.

Cell membrane was destructed and intracellular water molecules move between cells, resulting organ dysfunction.

Figure. 1: Hypothesis for freezing in variable magnetic field

We compared the existing freezing technology with the novel freezing technology to hypothesize the phenomena that occurred during the freezing procedure.

Rat ovary (left: after freezing in a variable magnetic field, right: after conventional freezing):

The 0.01%–0.1% egg-survival rate in the existing freezing method improved to 10%–20% when the ovary was frozen in a variable magnetic field.

Rat small intestine (left: after freezing in the variable magnetic field, right: after conventional freezing):

The decidual membrane and the intercellular spaces in the basement membrane that were observed after conventional freezing were rarely seen after freezing in the variable magnetic field.

Rat alveoli (left: after freezing in the variable magnetic field, right: after conventional freezing):

The alveolar architecture was damaged by the conventional freezing method, but it was preserved after freezing in the variable magnetic field. We also confirmed that the red blood cells in the capillaries were preserved after freezing in the variable magnetic field.

Porcine ovary (left: after freezing in the variable magnetic field, right: after conventional freezing):

Since porcine ovary and human ovary are more or less equal in porcine ovary was used for investigating the potential of ovary cryopreservation. Similar to the results for rat ovary, the cellular damage of porcine ovarian follicles was dramatically reduced.

Figure. 2: Tissue after freeze-thaw

Freeze-thawed tissue was HE-stained and histologically assessed.

treating pediatric cancer, particularly pediatric leukemia. However, “iatrogenic infertility” frequently occurs as a side effect of these procedures. Since the treatment outcomes for pediatric cancer are showing improvements, the quality of life (QOL) and maintenance of fertility after treatment have become social concerns. Since the current complete-remission rate is more than 70%, a treatment strategy for infertility must be immediately established with an aim to ensure QOL improvement.

Methods

Freezing experiment

Two Lewis female rats (250–300 g) were fasted for 24 hours and euthanized by ether inhalation. The organs were not removed from the body, which was directly used in the freezing experiments. One rat was frozen in a freezing equipment (ABI) that generated a variable magnetic field. The brine tank of the freezing equipment was filled with 60% ethylene glycol. The rat was enclosed in a nylon pack from which air was removed to the maximum possible extent; the nylon pack was placed in a weighted gauge and submerged in the brine tank.

At a magnetic-field intensity of 0.1–0.2 mT, the experimental material was cooled to 2 over a period of 1 hour and then cooled at the rate of $-0.5^{\circ}\text{C}/\text{minute}$ and frozen to -30°C . The entire cooling procedure, from the start of cooling to the completion of freezing, was performed in 2 hours and 45 minutes. The material was stored in an ultracold freezer at -80°C until it was used in the experiment. Another rat was used as the control and frozen in the ultracold freezer at -80°C for the same time period. After freezing, the animals were preserved for 24 hours and soaked in a saline solution at 30°C for rapid thawing.

Histological examination

We removed the heart, liver, lung, kidney, ovary, brain and pancreas from each rat. The organs were fixed in 10% formalin, and 4–5 μm thick serial sections were prepared from paraffin-embedded blocks by performing the usual procedures.

Results

Histological examinations were performed to determine the effectiveness of the magnetic-field-based freezing method for organ cryopreservation (Figure. 2). We obtained the following results for individual organs:

Brain: Injury to nerve cells decreased after cryopreservation in the magnetic environment.
Heart: While myocardial breakdown decreased after cryopreservation in the magnetic environment, cardiomyocyte adhesion was completely lost.

Pancreas: After cryopreservation in the magnetic environment, tissue breakdown in the Langerhans islet cells was slightly lower than that in the control.

Liver and kidney: Significant tissue destruction was observed after cryopreservation in the variable magnetic field.

Lung: Alveolar-structure breakdown was markedly reduced after cryopreservation in the magnetic environment, while alveoli were crushed after preservation in the ultracold freezer.

Small intestine: In the sample that was preserved in the ultracold freezer, substantial gaps were observed between tissues. However, the breakdown of the small-intestinal mucosal epithelium was reduced after cryopreservation in the magnetic environment.

Ovary: Follicular structure was better maintained after cryopreservation in the magnetic environment.

The extent of tissue breakdown was different between organs, but the results indicated that cryopreservation in the magnetic environment was more suitable for organ preservation.

Discussion

A Japanese company engaged in the development of food-freezing technology has devised a food-freezing technique, named "MRI, Magnetic Resonance Influenced freezing" that does not compromise on the quality of the thawed food. We studied the feasibility of using

Figure. 3: Procedure for cryopreservation of rats in a variable magnetic field:

We recorded the temperature of ethylene glycol in the brine tank (red line) and external air (blue line) during cryopreservation. The rats were fully cooled at 2°C and then frozen up to -30°C .

Figure. 4: Electromicroscopic photo of frozen Wasabi

The left photograph shows the cross-section of Wasabi frozen in the variable magnetic field. There were very few broken cellular walls, and there was no outflow of starch grains from the cellular walls. In contrast, the cross-section of Wasabi frozen by the existing technology, which is shown in the right photograph, shows outflow of starch grains from cellular walls due to cellular-membrane breakdown caused by the growth of ice crystals. The retention of taste after freezing was greatly influenced by the cryopreservation technology.

Figure. 5: Inhibition of osmotic-pressure gradient in the tissue

Water containing red food-coloring agents was frozen in the variable magnetic field (left photograph) and in the conventional freezer (right photograph). When the sample was frozen in the conventional freezer, the outer water molecules were frozen first, and therefore, the food-coloring ingredient was concentrated at the center. However, such ununiform concentration of the food-coloring ingredient was not observed after freezing in the variable magnetic field. The entire material was uniformly frozen.

this technique in organ cryopreservation. The results showed that this technique reduced tissue breakdown, especially in the brain, pancreas, small intestine, and ovary. When the small intestine was preserved in an ultracold freezer, ice crystals grew and melted in the tissue, creating a number of apparent spaces; however, ice-crystal formation was inhibited during cryopreservation in the magnetic environment.

The exact influence of the variable magnetic field on the cells is unknown, but we postulate that the entire supercooled organ may have undergone instantaneous freezing. As shown in Figure. 5, a solution of red food-coloring agent was uniformly frozen in the variable magnetic field. It is already known that a supercooled liquid undergoes instantaneous freezing with the addition of an ice nucleation or by vibration^{8,9)}, and the variable magnetic field may have produced an environment in which such a phenomenon was easily induced. Although no cryoprotectant was used in our study, it is necessary to develop an appropriate cryoprotective liquid for cryopreservation in the magnetic environment. As already mentioned, the duration of preservation varies from organ to organ, and the tolerability to cryopreservation also differs according to the organ. However, a cryoprotective fluid that fully penetrates the organ may cause cellular damage. Therefore, future studies should aim to develop a cryoprotective fluid that will not cause such damage. We aim to use pigs or monkeys, which have large organs, in cryopreservation research and use the results of those studies for clinical application in humans.

Differences between the significance of reproductive organs and other organs in cryopreservation research

In cryopreservation research, the goal of preservation of reproductive organs is significantly different from that of organs such as kidney and liver. Since the testis, ovary, etc., are cryopreserved for childbirth in the future, the objective is to retain a required number of living gametes in the organ. For example, the target success rate for obtaining a baby from the ooblasts in the ovary is 1/1,000,000. In contrast, complete success is expected in the case of organs such as kidney or liver, which are vital to human life. This is the difference between the target levels of success for cryopreservation research on reproductive organs and other vital organs.

Conclusion

Owing to the development of the immunosuppression regimen, organ transplant has become an established method for treating organ failure. Nevertheless, shortage of donors is a serious problem. Every year, an increasing number of organ-transplant candidates die due to the unavailability of donor organs.

If the organs can be preserved for longer periods in a good condition, more transplant candidates will be able to receive appropriate organs or select better-matched organs, thereby reducing the dosage of oral immunosuppressants.

Joseph Murray, a plastic surgeon, performed the world's first successful renal transplant between identical twins and was awarded the Nobel Prize for the development of the immunosuppressant azathioprine. His success was attained through close collaborations between internists, plastic surgeons, urologists, basic immunology researchers, and the pharmaceutical company. In the light of his example, our research team hopes to take on the challenge of developing innovative organ-cryopreservation technology by developing a close academic collaboration between plastic surgeons, who will play a central role, basic-science researchers, and the organ-cryopreservation company.

<Issue 1: The potential of cryopreservation in a magnetic environment>

There is no rational explanation for the effect of magnetic fields on the cells or organs.

<Issue 2: Development of protectants for organ cryopreservation:>

Since research on protective agents for cryopreservation of organs with heterogeneous cell populations is still in the primary stage, the results of these studies are awaited.

<Issue 3: Establishment of thawing technique:>

It is essential to establish a methodology for inhibition of recrystallization during the thawing of large organs.

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