Whole Ovary Cryopreservation Applying Supercooling under Magnetic Field

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Abstract

Chemotherapy and radiotherapy are performed for the treatment of cancer in children, especially leukemia. But, the side effects of these treatments are a problem of continuing concern, and improving the patient’s quality of life (QOL) following successful treatment remains a challenge. Especially with radiation therapy, disorders of reproductive function are considerable, and cases of infertility are extremely high. Currently, research on the cryopreservation of ovarian tissue is being energetically pursued, but because of factors such as reduction in number of egg cells and physical disruption due to tissue fragmentation, the successful fertilization rate of the thawed ovum has been extremely low. Accordingly, cryopreservation of the ovaries as an entire organ has been attempted, but this has yielded little success. This study focused on a method for cryopreservation through the application of supercooling under a variable magnetic field, and the development of a freezing system allowing for an arbitrary change in frequency of magnetic field applied to the specimen. To confirm the interrelation between a variable magnetic field and supercooling, physiological saline solution was frozen under a variable magnetic field, and the progress and stabilization of supercooling was verified for magnetic field frequencies from 200 to 200 kHz. Additionally, ovaries were frozen under magnetic field, and histological assessment of the tissue was performed.

Keywords

Ovarian cryopreservation, Supercooling, Variable Magnetic field, Mechanical engineering
Introduction

Treatment techniques for childhood cancers have progressed such that the 5-year survival rate for childhood leukemia has improved to 70-80%. However, the number of patients suffering hypogonadism or infertility due to chemotherapy or radiation therapy has also climbed to 97-98%. Due to physical damage and post-transplantation ischemic injury during the process of ovaries fragmentation, the fertilization success rate of the thawed eggs is extremely low, 2-5%, in spite of the many researchers enthusiastically pursuing research on cryopreservation of ovarian tissue. Therefore, research was undertaken on the freezing of, not only the ovarian tissue, but the ovaries as an entire organ. However, this met with little success. The point of issue was the fact that the entire ovaries could not be instantly frozen using liquid nitrogen, which was conventionally used for freezing, resulting in the growth of ice crystals, freeze concentration, and cell destruction.

Currently, attention is focused on a completely different freezing process in which the application of a variable magnetic field to the specimen safely brings about this supercooling phenomenon. The supercooling phenomenon is a state in which even when the temperature falls below the freezing point, a phase conversion from liquid to solid does not occur. By instantly freezing from a supercooled state facilitated by a variable magnetic field, freeze concentration is prevented, and cell destruction is inhibited. However, the correlation between a variable magnetic field and the supercooling phenomenon has not been entirely proven, and remains hypothetical. The primary parameters of a variable magnetic field are frequency and intensity, and in this study we focused mainly on frequency. The physiological saline solution freeze trial was performed with the frequency of the variable magnetic field as a parameter, to clarify the effective conditions for supercooling. Additionally, a freezing experiment was performed with the ovaries specimen under magnetic field, and histological assessment was made.

Method

**System for freezing ovaries**

The cooling system was a Stirling engine, cooling unit with an aluminum base and sides of ultrahigh molecular polyethylene surrounding a cooling tank (diameter: 50 mm, depth 70 mm) filled internally with ethanol. The cooling tank was covered in heat insulating material, and a coil was wound externally to it. Alternating current was provided with a function generator, and a magnetic field of $1.2 \pm 0.2 \text{ mT}$ was applied to the specimen within the coil. Since it was a concern that vibration from the Stirling engine would eliminate the supercooling effect, the specimen was fixed to a stand and kept out of contact with the cooling tank. An overview of the system is shown in Figure 1.

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**Figure 1.**

*System for freezing ovaries under magnetic field, system overview*

Stirling engine, Function generator, Coil, Ethanol, Thermocouple

It is known that a shielding effect exists whereby there is a markedly decreased intensity for a variable magnetic field that passes through a magnetic substance such as metal. For this reason, the cooling tank surrounding the specimen was constructed of nonmagnetic material (ultrahigh molecular polyethylene).
Physiological saline solution freezing experiment

As an indicator of the degree of supercooling, the absolute value of the difference between the freezing point and the temperature just before the resolution of supercooling was defined as the degree of supercooling (Figure 2). Magnetic field intensity was set to 0 or 1.2 ± 0.2 mT, a variable magnetic field of frequency 50 Hz, 200 Hz, 2 kHz, 20 kHz, or 200 kHz, was applied to the physiological saline solution (freezing point: -0.65°C), and it was cooled at a fixed rate of -1.8°C/min until supercooling resolved. Measurements were taken 15 times at each frequency.

Ovaries/liver freezing experiment

Three ovaries and one liver specimens were taken from pokers and cut into 4.5 mm squares, placed in physiological saline solution for 1 hour, and immersed for at least 2 hours in cryoprotective agent (10% final concentration trehalose (Wako) added to Leibovitz’s L-15 culture medium (Invitrogen). Thereafter, these were embedded in OTC compound, and the freezing experiment was performed utilizing the freezing system under magnetic field. Ovaries were frozen with no magnetic field applied, or with a variable magnetic field of intensity 1.2 ± 0.2 mT, and frequency 2 kHz, applied to the specimen. The ovaries were frozen with a slow freezing method (frozen with the same conditions as the physiological saline solution freezing experiment) and a rapid freezing method (direct immersion of embedded specimen in -60°C ethanol, and instantly frozen). Additionally, the temperature of the exterior of the freezing container was measured during the slow freezing to confirm any variation with the degree of supercooling. Rapid freezing only was performed on the liver specimen under identical conditions. The ovaries and liver were also frozen in dry ice/acetone without the use of cryoprotective agents as a control. All specimens were maintained at -80°C for 24 hours after freezing.

Histological assessment

The ovaries and liver, which were frozen under magnetic field, were prepared in 4-5mm cryosection using customary procedures, HE stained, and histologically assessed under microscopy.

Statistical analysis

Welch’s test was utilized to look for a significant difference in the average degree of supercooling of the physiological saline solution when the magnetic field frequency was varied. Significant difference was screened for with a one sided test at the 90% and 95% confidence intervals.

Result

Physiological saline solution freezing experiment

To 1 ml physiological saline solution, no magnetic field, and then a variable magnetic field with frequency 2 kHz was applied, and the degree of supercooling was measured (Figures 3, 4). This resulted in a significant acceleration of supercooling of physiological saline solution under magnetic field measured over the magnetic field frequency interval of 50-200 kHz, at 200 Hz, 2 kHz, 20 kHz, and 200 kHz, making clear the increase in degree of supercooling due to the application of a variable magnetic field. Moreover, because the standard deviation of the degree of

Figure 2.

Definition of supercooling phenomenon and degree of supercooling

Supercooling is an unstable phenomenon in which the temperature is brought below the freezing point but a phase change from liquid to solid does not occur. The degree of supercooling is defined as the difference between the freezing point and the temperature just before the resolution of supercooling, and the degree of supercooling was measured with this index.
magnetic field was small, it was suggested that the supercooling phenomenon contributed to stabilization as well.

**Ovary/liver freezing experimental results**

While the greatest increase in degree of supercooling occurred with the application of a variable magnetic field with a 2 kHz frequency, ovaries and liver froze with the slow freezing as well as the rapid freezing processes (Figures 5, 6).

However, there was no difference observed in the degree of tissue destruction in ovarian tissue for the controls or each set of conditions of freezing under magnetic field. Although, in the case of the liver specimens, there was suppression of sinusoidal expansion of capillaries between hepatic lamina for specimens rapidly frozen under variable magnetic field in comparison to the controls. In short, this suggested that tissue destruction due to freezing was suppressed.

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**Figure 3.**
Histogram showing degree of supercooling for physiological saline solution

The degree of supercooling frequency distribution is shown for the 15 trials of the physiological saline solution freezing experiment, with no magnetic field, and with a 2 kHz variable magnetic field. Compared with the no magnetic field condition (blue), the physiological saline solution with a variable magnetic field of frequency 2 kHz applied had a clearly heightened degree of supercooling, and the variance of the degree of supercooling was decreased.

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**Figure 4.**
Summary of the degree of supercooling of physiological saline solution (sample means, standard deviations)

The graph shows a summary of the means and standard deviations for the degree of supercooling at each frequency condition.

* A significant difference in population mean in comparison to the no magnetic field condition was shown using a one sided Welch’s test at the 90% confidence interval.

** A significant difference in population mean in comparison to the no magnetic field condition was shown using a one sided Welch’s test at the 95% confidence interval.
Frozen ovarian tissue
4-5 μm of swine ovarian tissue was frozen with no magnetic field applied as well as under magnetic field. It was HE stained and histologically assessed under microscopy.

Frozen hepatic tissue
4-5 μm of swine hepatic tissue was frozen with no magnetic field applied as well as under magnetic field. It was HE stained and histologically assessed under microscopy.
Discussion

Relationship between degree of supercooling and ice crystal growth

The freezing technique under magnetic field published in the work of Mihara et al. differed entirely from the liquid nitrogen and programmable freezers conventionally utilized for the freezing of internal organs\(^1\). Mihara et al. proposed stabilizing the supercooling with the application of a variable magnetic field to make possible the freezing of even large volumes of tissue while inhibiting tissue destruction. The correlation between a variable magnetic field and supercooling was entirely unknown, but it was shown that the relationship between the degree of supercooling and the volume of pure ice crystals could be expressed as a power function of time\(^2\). In short, in the freezing of internal organs, intra-and extracellular osmotic pressure variation due to freeze concentration could be inhibited by means of instant freezing from a high degree of supercooling, and we may hypothesize that this would inhibit tissue destruction. However, under actual conditions, supercooling is an extremely unstable, probabilistic phenomenon, and the supercooling state is eliminated by external factors such as impact and introduction of foreign bodies\(^2\). Therefore, it was considered to be exceedingly difficult to control. In this study we have shown, for the first time in history, that the variable magnetic field contributes to the facilitation and stabilization of supercooling. These experimental results are not only important to internal organ freezing research, but may play an important role in the study of the atomic/molecular properties of matter as well.

Ovum and hepatocytes size and freezing conditions

According to the liver freezing experimental results, a variable magnetic field was shown to be effective for the cryopreservation of internal organs, however this efficacy was not observed in the experiments with ovaries. The size of the ovum varies with its stage of maturity. Swine ovum size is 30-80 mm, while the hepatocyte size is 20mm.

Additionally, the differing results may be considered a manifestation of the difference in tissue structures such as the uniformity of hepatocyte cell composition or the abundant moisture content of the ovarian follicle. More detailed research is needed on, not only magnetic field intensity and frequency, but on factors such as freezing point and freezing speed as well.

Conclusion

In this study, we performed basic research on freezing under magnetic field as a method of freezing ovaries in order that reproductive function may be preserved in childhood cancer patients. This study has shown for the first time in history that a variable magnetic field contributes to the acceleration and stabilization of the supercooling phenomenon. Additionally, the efficacy of the variable magnetic field in hepatic freezing is suggested by the ovaries/liver freezing experimental results. Future progress in the identification of the optimum cooling conditions for each tissue type is essential.

Issue 1: Feasibility of cryopreservation under magnetic field

The basis for the efficacy of a magnetic field for the freezing of cells and organs is unknown.

Issue 2: Development of internal organ cryoprotective agents

Research on a cryoprotective agent for organs composed of various types of cells is still developing, and future research and development is awaited.

Issue 3: Establishment of a thawing method

In the freezing of internal organs of a large volume, a method of inhibiting recrystallization during thawing is required.
Reference

1) Mihara M, Nakagawa T, Noguchi S, et al:
2) Saito A, Ohkawa S, Une H:
   Research on outer factor affecting the freezing of supercooled water. JSARE, 1991;8(2):151-60. (in Japanese)
3) Inaba H, Takeya K, Nozu S:
   Fundamental study on continuous ice making using flowing supercooled water. JSME Int. J. Ser. B, 1994;
   37:358-93. (in Japanese)
4) Shichiri M, Araki Y:
5) Teraoka Y, Saito A, Ohkawa S, et al:

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Web Reference

- University of Tokyo, Academic Collaborations Society
  http://gakujutu.umin.jp/English/
- Women’s Clinic OIZUMIGAKUEN (in Japanese)
  http://www.reniya-womens.com