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Title
Subzero 24-hour non freezing rat heart preservation
  ·A novel preservation method in a variable magnetic field ·

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Abbreviations

1. MRI, Magnetic Resonance Influenced freezing
2. +dP/dt, the peak positive dP/dt
3. –dP/dt, the peak negative dP/dt
4. ATP, adenosine triphosphate
5. UW, University of Wisconsin cardioplegic solution
6. CAS, Cells Alive system
Abstract

Background:
A new supercooling system using a variable magnetic field has been recently developed. Subzero non-freezing preservation has been thought to be a beneficial method because of the lower metabolic rate. The purpose of this study was to evaluate the hemodynamic and metabolic effects of rat heart preservation in a variable magnetic field without cryoprotectants.

Methods:
Rats hearts were perfused *ex vivo* for 120 minutes after 24 hours’ preservation in two groups (n=6 each): (1) conventional storage group, in which the hearts were stored at 4°C, and (2) the subzero group, in which the hearts were preserved at -3°C in a variable magnetic field.

Results:
Reperfusion cardiac performance after preservation was significantly preserved in the subzero group compared with the conventional group with respect to heart rate, coronary flow, the peak positive dP/dt, and the peak negative dP/dt (p<0.05). Edema after reperfusion was significantly decreased (p<0.05), and the adenosine triphosphate level was higher in the subzero group (p<0.05).

Conclusions:
The rat hearts preserved in a variable magnetic field at -3°C showed better hemodynamic and metabolic performance than those preserved using conventional storage at 4°C.
Introduction

The limited availability of donor hearts is still a major problem in clinical heart transplantation. Currently, the safe ischemia time for a heart is limited to 5 or 6 hours of cold (2°C-4°C) ischemic storage. Extending the preservation time could increase the stock of donors and allow optimal immunologic matching of donors with potential recipients.

Preservation at a lower temperature may be advantageous, because the activity of enzymes shows a 1.5 - 2.0-fold decrease for every 10°C decrease according to van’t Hoff’s rule. Several organ preservation experiments at subzero temperatures have been reported using cryoprotectants such as polyethylene glycol, 2,3-butanediol, or anti-freeze protein. On the other hand, other groups have reported subzero organ preservation using a special supercooling refrigerator. However, the functional advantage of heart preservation in these refrigerators has not been reported.

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 the quality of the thawed food. In this method, the refrigeration chamber can generate a non-frozen condition below the freezing point, which is a so-called supercooled state, easily and stably, by applying a variable magnetic
field within the chamber. For organ preservation in medical transplantation, it is assumed that cooling to ice temperature results in reduction of organ metabolism, implying greater hypothermic preservation. Hearts preserved by supercooling would thus have better physiologic functions than those stored at 4°C. The aim of this present study was to evaluate cardiac physiologic functions after preservation by supercooling for an isolated perfused rat heart model.

Results

Heart rate

The heart rates at 30, 60, 90, and 120 minutes after reperfusion in each group are shown in Figure 3A. The heart rates in the subzero group (CAS) at 90 and 120 minutes were significantly higher than in the conventional storage group (4°C) (148.1±34.0 beats per minutes vs. 82.8±22.4 beats per minute at 90 minutes and 137.0±27.4 beats per minutes vs. 94.0±17.6 beats per minute at 120 minutes, p<0.05).

Coronary flow

The coronary flows at 30, 60, 90, and 120 minutes after reperfusion in each group are shown in Figure 3B. The coronary flows at 90 and 120 minutes were significantly
greater in the subzero group (CAS) than in the conventional storage group (4°C) (2.76±0.46 ml/min vs. 1.53±0.36 ml/min at 90 minutes and 3.26±0.66 ml/min vs. 1.48±0.37 ml/min at 120 minutes, p<0.05).

**Cardiac functions**

The peak positive dP/dt (+dP/dt) and the peak negative dP/dt (-dP/dt) are shown in Figure 4. +dP/dt and -dP/dt were significantly higher in the subzero group (CAS) than in the conventional storage group (4°C) at 30, 60, 90, and 120 min after reperfusion (+dP/dt: 289.1±154.6 vs. 32.4±25.3 mmHg/sec at 30 minutes, 361.5±222.0 vs. 53.9±24.6 mmHg/sec at 60 minutes, 333.5±220.5 vs. 70.5±23.6 mmHg/sec at 90 minutes, and 458.2±262.4 vs. 63.9±21.7 mmHg/sec at 120 minutes, p<0.05) (-dP/dt: -179.9±76.4 vs. -23.9±19.0 mmHg/sec at 30 minutes, -241.3±92.9 vs. -48.0±19.6 mmHg/sec at 60 minutes, -276.7±228.6 vs. 60.0±23.4 mmHg/sec at 90 minutes, and -351.1±247.6 vs. -52.2±15.9 mmHg/sec at 120 minutes, p<0.05).

**Tissue water content**

The tissue water content after reperfusion was higher in the conventional storage group (4°C) (82.6±1.2) than in the subzero group (CAS) (78.4±1.8; p<0.05; Figure 5A).
**Adenosine triphosphate (ATP) levels**

The ATP concentration after reperfusion was significantly higher in the subzero group (CAS) (245.8±40.0 μmol/g) than in the conventional storage group (4°C) (134.5±22.4 μmol/g) (p<0.05; Figure 5B).

**Discussion**

In this study, conventional heart preservation at 4°C and heart preservation in a subzero environment in a variable magnetic field were compared. From the present results for heart rate, dP/dt, and coronary flow, the subzero preservation group showed better preservation of cardiac function after reperfusion. The results for tissue ATP and tissue water content showed that the subzero preservation group also had better a preservation state with little edema. From this it is understood that subzero heart preservation in a variable magnetic field environment is promising for better recovery of metabolism and function than with conventional heart preservation at 4°C. This method enables subzero preservation without the use of cryoprotectants, eliminating the need for concern about their adverse effects. It is thought that future application of this method will make it possible to improve the state of heart preservation and extend
preservation time in heart transplantation.

In current clinical practice for heart transplantation, the heart is generally preserved for 4-6 h at 4°C. The advantages of the low temperature preservation generally used in experiments are the drop in metabolism and suppression of high energy store consumption. Metabolism is thought to continue until -60°C, but as seen when expressed as an Arrhenius plot, the cell metabolic rate is correlated with temperature. When temperature decreases 10°C, the metabolic rate roughly halves. Therefore, it may be considered that the drop in metabolism and suppression of high energy store consumption are greater with preservation at -3°C than with preservation at 4°C.

There are several reports of subzero preservation using cryoprotectants such as polyethylene glycol, 2-3 butanediol or anti-freeze protein with the aim of preservation at a lower temperature than conventional preservation at 4°C. These reports also state that organ preservation at lower temperatures has the advantages of attenuating enzyme activation and suppressing metabolism. To evaluate metabolism in the present experiment, it was decided to compare tissue ATP values, which are a well known indicator correlated to heart preservation status. Similar to previous studies, in the present experiment, tissue ATP was significantly preserved in the subzero preservation group, and it was demonstrated that tissue could be stored in a better
state than with conventional methods. In the clinical application of heart preservation using cryoprotectants, which have been used in experiments to take advantage of the metabolic inhibition effect of low temperature, there is the problem of adverse effects from the cryoprotectants themselves. On this point, since the heart preservation method of supercooling within a variable magnetic field enables subzero preservation without using cryoprotectants, it has the major advantage of eliminating the concern about adverse effects from cryoprotectants, making this method easier to apply clinically.

The freezing technology under a magnetic field environment such as was used in the present experiment was developed by a Japanese company engaged in the development of food-freezing technology, with the aim of cryopreservation that causes little cellular damage in food. There have been several reported studies to date similar to the present one on organ preservation in a subzero environment with the application of magnetic fields or voltage. In the report of Okamoto et al., 3,000 V were applied in preservation of a rat lung in a -2°C environment, and the preservation state 60 min after perfusion was reported to be better than with conventional lung preservation at 4°C. In a report by Monzen et al., a heart and liver were preserved for 24 hours and a kidney for 72 hours at -4°C with application of 100 V and 500 V, respectively. Enzymes that leaked
into the preservation fluid were measured, and the preservation state was reported to be better than with conventional preservation of 4°C. However, there are no reported evaluations of cardiac function after reperfusion for heart preservation using this technology. In the present experiment, since the peak positive dP/dt and the peak negative dP/dt measurement results were better with the supercooling heart preservation method in a variable magnetic field than with conventional preservation, it was demonstrated for the first time that systolic performance and diastolic performance are better maintained with supercooling than with conventional preservation. Combining the results for heart rate and coronary flow, it was also found that a better state of preservation was maintained than with conventional preservation for cardiac function.

However, preservation at subzero temperatures, while enabling metabolic inhibition and good preservation, is also reported to have the associated risks of freezing or breakdown of cellular homeostasis, which is maintained by Na-K ATPase activity. With respect to these risks, the present results showed high ATP levels and only mild tissue edema after reperfusion. These findings indicate that subzero heart preservation in a variable magnetic field environment can preserve hearts without causing the low temperature damage that is a potential concern.
Recently, preservation methods involving continuous perfusion 14, 15, 16 have been clinically applied 17 as a means of extending preservation time other than low-temperature preservation. However, these methods are somewhat complicated; for example, they use complex circuits and require a perfusion solution. In comparison, the present method of supercooling preservation in a variable magnetic field has the advantage of not requiring complex procedures such as a setup for continuous perfusion. Conversely, continuous perfusion preservation methods can wash out metabolic products and provide a constant energy source, thus providing an improved preservation state. Therefore, since the techniques and concept differ from the present method, it may be that by combining the two approaches an even greater improvement in the state of preservation can be expected. The ability to transport organs between countries for transplantation, which may become possible through further improvements to make this refrigerator portable, could also help transplantation medicine to spread more widely.

In conclusion, the results of the present study demonstrate that better preservation in terms of both cardiac function and metabolism, without causing low temperature damage, was possible with supercooled heart preservation in a variable magnetic field when compared with conventional preservation at 4°C in 24-hour preservation of rat
hearts. This method has the potential to become a revolutionary technique in transplantation medicine.

Limitations

This experimental model was Isolated heart perfusion model according to Langendorff apparatus with a Krebs-Henseleit solution. In the future, comparative investigations with blood such as heterotopic transplantation will be needed.

Technology for freezing in a variable magnetic field has the advantages of no concentration gradients and uniform freezing in both the outer and inner parts of food. Considering this, these techniques will probably be even more useful in the preservation of large solid organs, such as the heart and liver of large animals, than in small animals such as rats. In the future, comparative investigations with large animals will be needed. Furthermore, since allogeneic transplantation is conducted clinically, chronic experiments to compare the state following transplantation and other outcomes will be needed in the future.

Materials and methods
Healthy adult (10-12 weeks old, 250-300 g) Wistar rats were prepared. All animals received humane care in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996; Bethesda, MD). Each rat was anesthetized by inhalation of diethyl ether and intraperitoneal injection of pentobarbital (30 mg/animal). Each rat underwent mid-line abdominal and bilateral chest incisions, separating the anterior chest and the diaphragm, and was heparinized with 500 IU. The superior vena cava, inferior vena cava, and pulmonary artery were dissected, and 4 °C University of Wisconsin cardioplegic solution (UW; Via Span, Astellas Pharma Inc, Tokyo, Japan) was administered thorough the ascending aorta (total 50 ml, 5 ml/min). After that, the heart was preserved in a bath filled with UW.

Supercooling system

For the preservation of rat hearts by supercooling, a refrigerator named the Cells Alive system (CAS) (ABI Co., Ltd., Chiba, Japan) was used. This refrigerator was developed to maintain a supercooled state by applying a combination of multiple weak energy sources that cause water molecules in the material to vibrate, inhibiting crystallization to form ice. Since the entire material is frozen uniformly from outside to inside, the
water molecules are thawed in the same state as before freezing. As a result, cells can be maintained without broken when the food is thawed (Figure 1). This study focused on the ability of this technology to maintain a supercooled state, and a preservation experiment at -3°C without macroscopic freezing was performed (Figure 2).

**Experimental Groups**

There were two experimental groups: the conventional storage group (n=6), in which the hearts were preserved in a 4°C refrigerator, and the subzero group (n=6), in which the hearts were preserved in the -3°C Cells Alive System. The hearts in each group were preserved for 24 hours. The aorta of the preserved heart was connected to a standard Langendorff apparatus and perfused in a retrograde fashion at a constant pressure of 80 mmHg for 2 hours with a 37°C Krebs-Henseleit solution prepared in our laboratory (in mM: NaCl, 118; KCl, 4.7; MgSO₄・7H₂O, 1.2; CaCl₂・2H₂O, 2.5; NaHCO₃, 25; glucose, 11.0; KH₂PO₄ 1.2; pH=7.6) gasified with 95% O₂ and 5% CO₂.

**Functional measurement in the Graft**

Hemodynamic parameters were monitored using a 3-Fr. latex balloon catheter inserted into the left ventricle (LV) via the left atrium and connected to a pressure transducer
(VO1706TSPL03, Edwards Lifescience) placed at the equivalent height to the heart, in combination with a recording system (RM-6000, POLYGRAPH SYSTEM, NIHON KODEN CORPORATION). The balloon was inflated and equilibrated to give an end-diastolic pressure of 8 mmHg. LV pressure and time derivatives of pressure were measured during contraction (+dP/dt) and relaxation (-dP/dt) with the integrated data system UCO (UNIQUE MEDICAL Co., LTD, Tokyo, Japan), and heart rate was monitored. Coronary flow was measured in the volumetric cylinder at 30, 60, 90 and 120 minutes after reperfusion.

**Tissue water content**

Tissue water content (as a percentage) was determined by the difference in wet weight and dry weight divided by wet weight and multiplied by 100%. After 120 minutes of reperfusion, the hearts were dried to a constant weight at 85°C for up to 48 hours.

**Determination of adenosine triphosphate (ATP) levels**

After 120 minutes of reperfusion, the hearts were immediately immersed in liquid nitrogen (-196°C) and stored frozen at -80°C until biochemical analysis. ATP content was expressed as micromoles per gram of dry weight.
**Statistical analysis**

All data are expressed as mean value ± standard deviation. The data were evaluated by the Mann-Whitney U-test for 2-group analysis. A value of p < 0.05 was considered significant. All statistical analyses were performed using SPSS for Windows version 16.0 software (SPSS, Chicago, IL).

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References


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Heart preservation using continuous ex vivo perfusion___ [Circ J_ 2007] - PubMed - NCBI.htm
**Figure Legends**

Figure 1
Concept of freezing in a variable magnetic field
(A) When the organ is expose to a combination of multiple ultraweak energies, water molecules in the organ are vibrated, ice crystallization of water content is inhibited, and the supercooled state is maintained.
(B) When the organ is directly expose to cold air (in a conventional cooling system), ice crystallization is initiated, and the ice on the surface obstructs the freezing of the internal layers, thereby causing multilayered freezing. The water molecules in the internal unfrozen segment are mobilized and sucked up toward the core of ice on the surface by capillary phenomenon.

Figure 2
Myocardial temperature in the conventional storage (4°C) and a variable magnetic field (-3°C, CAS)

Figure 3A
Heart rate during reperfusion
The heart rates are significantly higher in the subzero group (CAS) than in the conventional storage group (4°C) at 90 minutes and 120 minutes after reperfusion (*p<0.05).

Figure 3B
Coronary flows during reperfusion
The coronary flows are significantly higher in the subzero group (CAS) than in the conventional storage group (4°C) at 90 minutes and 120 minutes after reperfusion (*p<0.05).

Figure 4
Changes in The peak positive dP/dt (+dP/dt) (A) and the peak negative dP/dt (-dP/dt) (B)
+dp/dt and -dp/dt are significantly higher in the subzero group (CAS) than in the conventional storage group (4°C) (*p<0.05).

Figure 5A
Adenosine triphosphate (ATP) levels in the myocardial tissue at the end of 120 minutes’
reperfusion

The ATP level is significantly higher in the subzero group (CAS) than in the conventional storage group (4°C) (p<0.05).

Figure 5B
Tissue water contents at the end of 120 minutes' reperfusion

The tissue water content is significantly lower in the subzero group (CAS) than in the conventional storage group (4°C) (p<0.05).
(A) Cooling and freezing
In a variable magnetic field

(B) Conventional thawing and cooling technology