Preservation of myocyte structure and mitochondrial integrity in subzero cryopreservation of mammalian hearts for transplantation using antifreeze proteins—an electron microscopy study

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Abstract

Objective: Freeze tolerant fish and insects in nature are able to survive subzero temperatures by noncolligatively lowering the freezing temperature of their body fluids using a family of thermal hysteresis proteins (antifreeze proteins, AFPs) specific for each species. Past efforts to cryopreserve mammalian hearts using these proteins were unsuccessful. We report the first successful subzero cryopreservation of rat hearts using fish derived antifreeze proteins with preservation of myocyte structure.

Methods: Heterotopic heart transplantations were performed in isoimmuninc Sprague Dawley rats. Donors' hearts were arrested using University of Wisconsin (UW) solution and preserved in UW solution containing AFP I (six experiments) or AFP III (six experiments) at concentrations of 15–20 mg/cc for 2–6 h at subzero temperatures ranging from −1.1 to −1.3 °C. Four control experiments were performed by preserving harvested hearts in UW solution alone at −1.3 °C for 6 h. In all experiments ice was added in the solution for crystallization. Heterotopic transplantations were performed in the abdomen of the recipient rats. Viability was visually assessed and graded on a scale of 1 (poor contraction) to 6 (excellent contraction). The hearts were then fixed in vivo and processed for electron microscopy study.

Results: All hearts preserved at subzero temperatures using AFP I or AFP III survived displaying viability scores of 4–6 1 h after transplantation. Three of the four control hearts that were preserved at −1.3 °C without the protective effect of AFP froze and died upon reperfusion. Electron microscopy study of hearts preserved with AFP demonstrated preservation of myocyte structure and mitochondrial integrity.

Conclusion: Subzero cryopreservation of mammalian hearts for transplantation using AFP I or AFP III is feasible with preservation of myocyte structure and mitochondrial integrity.

Keywords: Heart; Preservation; Subzero; Antifreeze protein; Fish

1. Introduction

Heart transplantation has become an established surgical technique for the treatment of end stage heart failure. With current preservation techniques the ischemic time of a donated heart is limited to 5–6 h [1–3]. This does not provide sufficient time for optimizing donor–recipient tissue matching and causes significant logistic difficulties in the supply of organs from donors to recipients across distance.

Most of today’s preservation techniques employ hypothermic temperatures to inhibit metabolism and thereby inhibit ischemic damage. Theoretically lowering preservation temperature by 10 °C will lower the cells’ metabolic demand by approximately 50% [4]. The generally accepted method of preserving donor heart integrity is to arrest it with cold (4 °C) cardioplegic solution and then store it in an electrolyte solution immersed in ice [2,3]. Temperatures attained by this technique are believed to reach 4 °C [3]. Prolonging ischemic times by lowering the temperature below 0 °C was not studied extensively since the expected
damage to the heart was great and efforts were directed towards optimizing the storage media at \(0\)–\(4\) °C.

Freeze tolerant fish and insects in nature are able to survive subzero temperatures by noncolligatively lowering the freezing temperature of their body fluids using a family of thermal hysteresis proteins (antifreeze proteins, AFPs). Arctic fish start to produce AFPs once winter begins and the ambient temperature of \(0\) °C is reached, they survive sea water temperatures as low as \(-1.8\) °C with the presence of ice crystals without freezing as a result of the protective effect of the AFPs which lower the freezing temperature of their body fluids without altering its osmolarity. Previous efforts to cryopreserve cardiomyocytes or isolated hearts at subzero temperatures using AFPs were unsuccessful [5, 6].

We report, for the first time, successful cryopreservation at subzero temperatures of mammalian hearts transplanted heterotopically using antifreeze protein I (AFP I) derived from the arctic fish Winter Flounder and antifreeze protein III (AFP III) derived from the arctic fish Ocean Pout, demonstrating by electron microscopy preservation of myocyte ultrastructure.

2. Materials and methods

Heterotopic heart transplantation were performed in isoimmunnic Sprague Dawley rats weighting 250 g using the technique described by Ono and Lindsey [7]. The animals received humane care in compliance of the ‘Principals of Laboratory Animal Care’ and the ‘Guide for the Care and Use of Laboratory Animals’ prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH publication no. 86-23, revised 1985). Anesthesia was obtained by an intra-peritoneal injection of Ketamine (80 mg/kg) and Xalazine (8 mg/kg).

2.1. Heart harvesting

The donor rat underwent a midline abdominal and chest incision separating the anterior chest and the diaphragm; the anterior rib cage was hinged, exposing the heart. University of Wisconsin (UW; Viaspan, DuPont) cardioplegic solution, with heparin, was administered through the harvested hearts were preserved in UW solution alone at \(-1.3\) °C. The freezing temperature of a solution is determined by its osmolarity, UW solution freezes at \(-0.6\) °C. In order for the freezing to occur nucleating agent, which causes crystallization (ice, dust) or stirring or shaking of the solution are needed. AFPs prevent ice formation at subzero temperatures in the presence of nucleating agents without affecting osmolarity. A nucleating agent was introduced in all our solutions in order to ensure ice formation and freezing.

2.3. Heterotopic transplantation

In the recipient rats a long abdominal incision was used to expose the abdominal aorta and the inferior vena cava just below the renal vessels, to the point chosen for the anastomosis. Rewarming of the donor heart is passive and occurs while the heart is transplanted at room temperature similar to the techniques used in clinical heart transplantation. The donor’s heart ascending aorta was anastomosed to the recipient’s abdominal aorta and the donor’s heart pulmonary artery was anastomosed to the recipient’s inferior vena cava. Viability of the transplanted heart 1 h following transplantation was visually assessed and graded on a scale of 1 (poor contraction) to 6 (excellent contraction).

2.4. Pathological evaluation

Transplanted hearts were kept in the recipient animal for 60 min before histopathological and electron microscopy evaluations were performed. After each experiment, the hearts were fixed in vivo while beating using a 2% paraformaldehyde and 2.5% gluteraldehyde solution at room temperature for 1 h, and post-fixed for an additional hour with 1% osmium tetroxide at 4 °C, dehydrated in alcohol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and photographed using JEOL 1200EX transmission electron microscope.

3. Results

Overall 16 heterotopic heart transplantsations were
performed: six experiments were performed using AFP I, six experiments using AFP III and four control experiments (Table 1). All 12 hearts preserved at subzero temperatures using AFP I or AFP III survived while three of the four control hearts that were preserved at $-1.3 \, ^\circ\text{C}$ without the protective effect of AFP, froze and died upon reperfusion. Viability scores of the hearts preserved with AFPs ranged from 4 to 6 (Table 1).

Electron microscopy studies of hearts preserved at subzero temperatures with AFP I or AFP III demonstrated preserved myocyte ultrastructure and mitochondrial integrity (Fig. 1a, b), while subzero preservation without AFP protection resulted in disruption of Z lines, destruction of myocyte ultrastructure and mitochondrial swelling and destruction (Fig. 2a, b).

4. Discussion

Scholander et al. [8] were the first to demonstrate that fish can survive in sea water at temperatures as low as $-1.9 \, ^\circ\text{C}$. DeVries and Wholschang [9] isolated from the blood of Antarctic notothenioid fish a proteinaceous macromolecule responsible for this ‘antifreeze’ activity. A variety of antifreeze peptides (AFPs) have been isolated and are classified broadly into six main groups according to their chemical structure: antifreeze glycoproteins (AFPG), four types of AFPs (AFP I–IV), and two types of insect derived AFPs [10].

AFP I is a 3.3–4.5 kDa alanine rich protein that is found in the Winter Flounder. It has a tertiary structure of a single $\alpha$ helix and it lowers the freezing temperature of a solution by 0.8 $^\circ\text{C}$ [11]. AFP III is a globular protein weighing 6.5–14 kDa consisting of short $\beta$-strands with a single turn of $\alpha$-helix in the loop region and is found in the Eel pout, Ocean Pout and the Wolf fish [11,12]. AFP III lowers the freezing temperature of an aqueous solution by 1.2 $^\circ\text{C}$.

AFPs are believed to have three distinct mechanisms of action that are responsible for biological protection at subzero temperatures: AFPs depress noncolligatively the freezing temperature of aqueous solutions (by three orders of magnitude more than their osmotic contribution) presumably due to their ability to bind to ice crystals [12], they inhibit recrystallization of ice [11,12], and they inhibit ionic leakage through biological membranes at low temperatures when phase transition occurs [13–15]. The effect of AFPs on tissue cryopreservation is controversial.

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<th>AFP I</th>
<th>AFP III</th>
<th>Control</th>
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<tr>
<td>AFP conc. (mg/cc)</td>
<td>Temperature/time ($^\circ\text{C}$/h)</td>
<td>VS</td>
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<tr>
<td>20</td>
<td>$-1.1/2$</td>
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AFP conc., antifreeze protein concentration; VS, viability score on a scale of 1–6 (0, dead); Temperature, preservation temperature.
AFPs have been reported to protect single mammalian cells and embryos at subzero temperatures. Examples include cryoprotection of immature oocytes and two-cell embryos of mice and pigs [16,17], bovine and ovine embryos at morula/blastocyst stage [18], and cryoprotection of chimpanzee sperm [19]. Conversely, several experiments cast a doubt on the cryoprotective effect of AFPs, as no benefit has been demonstrated by addition of AFPs to cryopreserved equine embryos [20] and human red blood cells in glycerol [21].

Cryoprotection of solid organs using AFPGs has been performed on rat livers and hearts. Successful isolated rat livers subzero preservation has been performed by Lee et al. [15]. Rat livers perfused with AFPG in Krebs solution before hypothermic storage had a higher rate of bile production and less enzyme leakage upon reperfusion compared with livers not perfused with AFPG. Whole rat livers frozen to −3 °C protected by glycerol and AFPG had increased bile production and less hepatocyte structural damage compared with livers preserved with glycerol alone [22]. Cryopreservation of cardiomyocytes or hearts using antifreeze proteins has been unsuccessful. Mugnano et al. [5] examined the effect AFPG on freezing (−4 °C) of cardiomyocytes. With the use of cryomicroscopy, they demonstrated that in the solution frozen without AFPG large blunt crystals were formed excluding most cardiomyocytes from the plane of ice formation. After thawing cells appeared similar to unfrozen cells, Spicular ice formed rapidly in the 10 mg/ml AFPG solution. The needle-like crystals appeared to penetrate the cardiomyocytes, resulting in intracellular freezing followed by cell lysis.

Wang et al. [6] evaluated subzero cryopreservation of rat hearts using the Langendorff in vitro model of working isolated rat hearts. Cardiac explants were preserved using AFPG at different concentrations at subzero temperatures of −1.4 °C. Hearts that were preserved for 3 h at concentrations of 10 mg/ml AFPG failed to beat upon reperfusion. The authors found that AFPGs were deleterious to the isolated rat hearts in a dose-dependent manner exacerbating the damage caused by freezing.

Our study is the first to demonstrate successful subzero cryopreservation of mammalian hearts using AFP I and AFP III in an in vivo heterotopic heart transplantation model. All hearts preserved at subzero temperatures using AFP I or AFP III survived displaying good to excellent viability scores. We believe that the key to our success is maintaining preservation temperatures that do not allow freezing. Once freezing occurs the heart is irreversibly damaged. Preservation target temperatures and AFP I and III concentrations in our experiments were determined according to preliminary experiments, in which AFP thermal hysteresis activity curves in UW solution were plotted, in order to achieve a supercooled solution without freezing. The electron microscopy studies, which demonstrated that antifreeze proteins prevented the damage caused by freezing, correlated well with the post-transplant hemodynamic performance.

We are aware of the subjective manner in which the viability of the transplanted hearts was evaluated in our study. However, in previous preliminary studies viability scores of heterotopically transplanted rat hearts were graded by three independent observers and were found to be identical.

We conclude that subzero cryopreservation of transplanted mammalian hearts using AFP I or III is feasible. Further ongoing studies will determine whether subzero cryopreservation can prolong ischemic times beyond 6 h with better post-transplant performance.

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proteins were provided as courtesy from A/F Protein, Waltham, MA, USA.

References


Appendix A. Conference discussion

Dr C. Heilmann (Freiburg, Germany): I have a question regarding your first talk. Are AFP I and III intracellular proteins or are they in the serum? And if they are intracellular, how might they work if you only perfuse the heart?

Dr Amir: The proteins are extracellular. They prevent freezing by binding to ice crystals and preventing them from forming as large crystals. Now, part of the damage that was attributable to the antifreeze proteins was that they blocked ion channels on the membranes, because they cover them. We overcame this problem by rinsing them out and keeping them only in the solution, the preservation solution. They are extracellular. There are 6 or 8 types of antifreeze proteins, and there are many subtypes, which differ in their molecular weight and structure.

Dr C. Yankah (Berlin, Germany): During the thawing procedure, how long do you have to take to remove the antifreeze proteins?

Dr Amir: Five minutes. I rinse them out. We had a lot of bad experiments when I started with this. And it took us about a year to find out that if you tried to rinse them with room temperature solution, it doesn’t work. Only when you rinse it in cold solution, it works. The antifreeze proteins change their configuration at low temperatures so only cold rinsing enabled us to perform successful transplantations.

Dr Yankah: Is it toxic when the temperature is higher? Dimethyloxide as a cryoprotectant for the homografts, for example, needs to be diluted immediately beyond 4 degrees, otherwise it becomes toxic at a higher temperature. I don’t know whether it applies to the antifreeze proteins as well.

Dr Amir: Well, this question is very controversial for us. We are performing also Langendorff experiments and we found out that sometimes they kill the heart and sometimes they don’t kill the heart. So I don’t know what the answer would be if they are toxic.

These proteins are not good for freezing. When the solution freezes, they change the morphology of ice crystals, make them spiculated, they are like knifes. And they just cut and destroy the tissue. The whole idea is to keep the solution from freezing and get it under zero degrees in order to prolong preservation.

Dr J. Martin (Freiburg, Germany): I was fascinated of this concept, increasing the intracellular glucose concentration using glucose and saline. It is known that the wood frog has special membrane glucose transporters to increase the intracellular glucose concentration. My question to you is, were you able to measure the tissue glucose concentration in your experiments and have you also measured the intracellular potassium concentrations?

Dr Amir: No, we didn’t measure neither the intracellular glucose nor potassium concentration. I don’t know how much gets in. But as we have seen from the antifreeze experiments, what’s important is the outside, there is no freezing. Once there is no nucleation outside, the heart doesn’t freeze. Because nucleation occurs where most of the fluid is. So if we prevent freezing outside, the heart won’t freeze.

Dr Martin: But you wouldn’t expect that intracellular glucose concentration would be increased in your experiments?

Dr Amir: I expect it will increase. I think it increases. And it gives us more protection. Furthermore glucose and insulin has proved beneficial in heart preservation and in cardioplegias, so it can just be helpful.

Dr E. Gamms (Dusseldorf, Germany): The entire freezing proteins you are using comes from the fish. And you are using it in rats. I mean the old idea of having completely different species doesn’t play a role, because you purify the protein that well, or what is the reason for that?

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1 This discussion also encompasses another paper presented at the EACTS Monaco Meeting.
Dr Amir: The protein is purified. We had some batches that were not very well purified and we had bad results. It took us a couple of months to find out that the batches were bad. We’re using drugs and medications that are derived from animals in our clinical setting, so I don’t see a problem in that. Antifreeze protein I is a very small protein, it weighs 4 kD. Antifreeze protein III is a protein that weighs 14 kD. It is just proteins. But it can cause allergic reaction. We’re using it only for preservation. We’re not giving it to the host, and we’re rinsing it afterwards.

Dr J. Vaage (Stockholm, Sweden): What is the point of this, going to subzero temperatures? I mean going, for instance, from plus 4 to minus 1, what does it add? Are you getting into the situation that everything stops so you can keep them and wake them up in 50 years, or what?

Dr Amir: Well, the point of it is to prolong ischemic times. Going down from 4 degrees to minus 1 degree gives us about 30% to 40% more time. The last slide is of preliminary studies. We have shown that after 18 hour preservation hearts that were kept at 4 degrees Celsius died while those that were preserved at −1.3 survived and had good viability scores. Using antifreeze protein, I can preserve rat hearts for 24 and even 28 hours. So it gives us 30% more of time.

Dr Vaage: But it’s still slow metabolism going?

Dr Amir: Yes. If you want to stop it completely, you have to go to minus 30, minus 40.

Now, the whole idea is to find a way to combine the modalities, either have glucose and insulin and a little bit of antifreeze protein and go down to minus 5, without freezing, and that would give us more time. Because the lower you get, the more time you have. And this is what we’re aiming for.