EXHIBIT 8

21st Century Medicine, Inc.

Summary/Proposal for Life Extension Foundation Supported Research 2005 - 2006

LEF Annual Report
&
Grant Letter

Doc. No. 0069

October 18th, 2006

To: The Life Extension Foundation

Re: Application for Grant Support for Year 2006 Research

Dear Sirs:

The purpose of this letter is to provide a summary of research planned and conducted at 21st Century Medicine during 2005 and 2006, and to request continued grant support for new and ongoing research projects of 21st Century Medicine, Inc. The primary mission of 21st Century Medicine remains to develop and deploy new technology and products in the fields of cryopreservation and ice control, and particularly to develop successful methods for the cryopreservation of challenging complex systems.

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1. Summary of research completed during 2005

Note: For the convenience of the reader in rapidly finding information in this letter, **bold text** is used to highlight the central points being discussed in each paragraph.

A) Brain Vitrification

Brain Slice Cryopreservation

The overall aim of the brain slice project is unchanged and is to demonstrate optimal methods for the cryopreservation of rabbit hippocampal slices by vitrification. The availability of perfectly preserved brain slices would be of significant value to the pharmaceutical industry in facilitating drug screening and reducing the costs of drug development. In addition it will provide valuable insights on whole brain preservation and, ultimately, suspended animation.

Studies focused on recapturing the success initially observed in our VM3 series. A systematic series of experiments was devoted to **"debugging" the protocol for adding and removing VM3**, using recovery of electrical responsiveness as the key end-point, although we continued to measure the K^+/Na^+ ratio as a supplemental test.

Lowering the temperature of exposure to VM3 from -10°C to -14°C seemed to be helpful, but was not sufficient.

Surprisingly, **basic investigations of the tolerance of these slices to osmotic changes** indicated that they were far more sensitive to prolonged hypertonicity than to hypotonic exposures. This observation led us to try reducing the concentration of mannitol used in the VM3 washout steps. Again to our surprise, and in seeming contradiction to the Harbor-UCLA experiments, better results were obtained when mannitol was reduced from 300 mM to 100 mM, and still better results were obtained when no mannitol was used at all. This strongly confirmed **that hippocampal slices are sensitive to prolonged shrinkage** but are not particularly sensitive to swelling, the reverse of what holds true for most cells.

Since each step of adding cryoprotectant involves transient shrinkage of the tissue, we sought to limit this shrinkage by using **a continuous-gradient, pump-driven system** for part of the cryoprotectant addition and washout process rather than using discrete concentration steps. This change in procedure **significantly improved our results**, giving reasonable recovery of electrical activity and essentially complete recovery of K^+/Na^+ ratio. However, we were still frustrated by the fact that some slices failed to respond at all while others may be able to respond reasonably well but not entirely up to control standards. Further consideration of the method showed that the pumping system used, while better than stepwise methods, actually produced the worst possible curve of concentration vs time, concentration rising rapidly and then more slowly. To determine whether further gains could be obtained, we tested a program of manual re-setting of the pump speed according to a predetermined schedule so that concentrations would rise slowly at first and then more rapidly, and, on washout, would fall rapidly at first and then more slowly. The continuous gradient system not only reduced osmotic stress but also reduced the exposure of the slices to air and reduced slice handling variations. **This final maneuver dramatically improved our results. Our VM3-treated slices were able to respond electrically as well as control slices, with K^+/Na^+ ratios also equivalent to controls.** Both the quality of the electrical responses and the number of responding slices were fully equal to 100% of control.

These results demonstrate that damage caused by VM3 exposure to date has really been the result of avoidable osmotic stress. This provides us with **very valuable and far from obvious proprietary information** that will give us a competitive advantage over any possible competing brain slice vitrification group. Specific patent protection for this technique may be appropriate.

Changes in apparatus were also made in order to carry out the continuous gradient system for adding and removing VM3. In our original non-gradient method, a slice carrier apparatus was using consisting of a single well 2 cm in diameter. Slices were positioned on a mesh floor at the bottom of the carrier and were transferred from container to container for loading and unloading. For the modified method, slices were held in 2-3 vertically stacked slice decks in a larger tube. Each deck carries 4 -6 slices.

Before the end of 2005, using an accelerating dC/dt curve instead of a decelerating one to load cryoprotectant and using a new vitrification apparatus, we vitrified slices at -135°C and held them at that temperature for 10 min. When these slices were rewarmed we were able to obtain, for **the first time in history, good electrical responses in vitrified/rewarmed hippocampal slices.** We also showed **that slices could be stored for 2-7 days at -135°C with good electrical activity after rewarming.**

By the end of 2005, 70% of responsive slices were able to generate 80% of the control electrical responsiveness after vitrification and rewarming.

We also began the design and construction of a computer-controlled system for adding and removing cryoprotectants to reduce variability in our results.

A) Brain Vitrification

Whole Brain Cryopreservation

Studies focused on alternatives to M22, the origin of ultrastructural damage associated with cryoprotectant washout, freeze-substitution, mechanisms of observed tissue edema, and greatly expanded studies of brain cold storage by various means.

Alternatives to M22. The most promising replacement for M22 in 2005 was **MEG**, and another promising candidate was **E22.** These solutions are less toxic than M22 based on numerous kidney slice experiments, and it was hoped they might also better penetrate through the blood brain barrier. Both 100% and 81% of full-strength MEG were perfused, and so was 100% E22. Analysis of the results is still pending.

Because of the rather amazing observation that 81% of full-strength M22 allowed the brain to be vitrified and rewarmed without ice artifacts, we also considered the much more dilute **VM3** solution currently being studied in the brain slice model. VM3 is an 8.4M solution, and 81% M22 is a 7.6M solution like VS4, so VM3 should be both fully sufficient for brain vitrification and dramatically safer and more convenient to use than M22 and might produce less shrinkage injury than M22. Ultrastructural images after VM3 perfusion or perfusion plus vitrification and rewarming were obtained in 2006 and analysis is still pending.

Some experiments were also carried out with **7.5M glycerol**. This agent caused **extreme shrinkage of the hippocampus** because of the existence of the blood-brain barrier, but less shrinkage of the brain overall than M22. To study its possible use for vitrification in whole body cases, we devised a method for cooling rabbit cephalons at 0.15°C/min, the rate attainable in human whole body cases. Adding Dacron wool to the inside of the beverage cooler used in our 0.3°C/min studies resulted in **a cooling rate of around 0.15°C** per minute. Despite cooling to below Tg at this very low rate, **only a small number of ice crystal artifacts were seen** after rewarming and fixing these brains. Glycerol is known to be remarkably non-toxic for the brain, and deserves consideration as a future component of a brain vitrification solution. However, initial ultrastructural observations **did not replicate the pristine look of the classic canine 7M glycerol experiment**, and glycerol perfusion resulted in massive superficial edema of the cephalon.

We also began more detailed examination of the ability of **3-O-methyl-rac-glycerol** and a **relatively novel cryoprotectant** we are further developing to cross the blood brain barrier and enter brain cells. We also **contemplated the design of a specialty cryoprotectant** specifically tailored for rapid uptake by the brain.

Origins of ultrastructural damage associated with cryoprotectant washout. The most obvious problem we currently see for recovering brain viability after cryoprotectant perfusion (other than the passage of time in the cold by itself, as discussed below) is the fact that we cannot currently remove vitrifiable levels of cryoprotectants without observing major ultrastructural damage. **If we are to be able to address this problem, we must first find out its origin.** If the damage is caused by re-expansion of

the brain after shrinkage, how much shrinkage is permissible? Does it matter if permeating agents are present, or is the damage related to shrinkage regardless of the cause? Is the damage related to toxicity, so that lower concentrations don't produce the damage even if they cause almost the same amount of shrinkage as higher concentrations? If the injury is caused by re-expansion after shrinkage, how much re-expansion produces the injury, and would modifying the washout just before this point is reached allow the injury to be eliminated or reduced?

To begin to answer some of these questions, **the following experiments were done**. First, some brains were perfused with low concentrations of cryoprotectant, then perfused free of cryoprotectant and fixed. Second, some brains were perfused with 0.3M concentrations of sucrose and glucose to verify that they also severely shrink the brain so that the results of washing out these agents can be compared to other shrinkage-re-expansion protocols. (Both glucose and sucrose did shrink the brain, but, interestingly, some glucose actually entered the brain and entered the brain cells based on the histological observation of shrinkage spaces surrounding neurons in brains perfused with sucrose but not with glucose. This observation suggests the possibility of using at least some glucose as a cryoprotectant for the brain.) Third, some brains were perfused with full-strength M22 and partially washed out and fixed. Fourth, some brains were perfused with VM3 and all of the VM3 was washed out, to see if the lower toxicity and lower concentration of VM3 might avoid the injury. **Ultrastructural results were not yet in for any of these experiments as of the end of 2005.**

Freeze-substitution studies. Freeze-substitution is a technique that allows ice to be visualized in frozen systems. It is of present interest for **checking the ability of brains perfused with VM3 to remain truly ice free near dry ice temperature**, versus simply being ice artifact free on warming. We assume the latter implies the former, but it is prudent to check this assumption. We have now used freeze substitution to check for the presence of ice in VM3-perfused brains under three different conditions, and the results are presently being analyzed.

Mechanisms of edema development. When we washed VM3 out of rabbit cephalons, **a surprisingly severe degree of edema was observed in the superficial tissues**. Because VM3 contains 7% PVP and because VM3 was found to be much more damaging to rabbit kidneys than VMP, which lacks this PVP, we assumed it was probably the PVP that resulted in the observed edema. If so, this effect has to be produced by the simultaneous presence of the other components of VM3 because in the absence of cryoprotectant, cephalons perfused with LM5 plus PVP were no more edematous, based on their weight, than cephalons perfused with LM5 only. Therefore, **the origin of the observed edema remains unexplained**. If we decide to pursue studies of VM3, we will revisit this issue.

Electrical responses and structural integrity in brain tissue after lengthy cold perfusion or static cold storage. The problem of **rapid brain deterioration in the cold,** whether during static storage or during continuous perfusion, was investigated using histological, ultrastructural, and neurophysiological endpoints. Brains were perfused with RPS-2, LM5 + HES, RPS-TES, and B2, stored for 24 hours at 0°C, and fixed. Results for B2 are not available, but of the other solutions, **RPS-TES was the best**. Ultrastructural results for RPS-2 showed **intact cell membranes but no intracellular contents after 24 hours of cold storage**, although synapses and myelin appeared to remain intact. Other brains have been perfused with B2, stored one hour, and **sliced so that the electrical responsiveness of the slices could be measured using Dr.**

Tan's standard method. One hour of cold storage in B2 was compatible with 74% recovery of control slice electrical activity and complete recovery of K^+/Na^+ ratio. However, **3 hours of cold storage reduced electrical activity to just 45% of control, and 5 hours or 24 hours of storage resulted in no electrical responses.** The texture of the brain was found to become progressively firmer with time in B2, making it hard to cut slices at 24 hours of storage.

Continuous perfusion at 3.5°C preserved the ability of the hippocampus to respond electrically better than did simple cold storage in situ. Using a perfusate (B4) that did not permit any electrical response to be obtained after 3 hours of static cold storage, **continuous perfusion at 3.5°C allowed electrical responses to be recovered after 5 hours of cold preservation.** Evidently, continuous perfusion can usefully support brain metabolism even at 3.5°C, a result consistent with canine hypothermia experiments involving continuous perfusion versus circulatory arrest.

Brain cold storage using Critical Care Research (CCR) neuroprotective cocktails. We perfused a rabbit cephalon with cold B5 brain perfusate containing CCR's water soluble protective agents until the temperature reached 5°C, and then the cephalon was stored statically for 3 hours and checked by Dr. Tan's slice assay. In a second experiment, most of CCR's water insoluble agents were tested in place of the water soluble agents in the first experiment. A third experiment was done the same way but without including any of CCR's agents in the cold perfusate. The results showed that without CCR's agents, perfuse-cooling with B5 allowed a small amount of activity to be observed, **but both the water soluble agents and the water insoluble agents electrically silenced the brain.** We think these agents take longer to wear off than the time scale of our experiments, which might in fact relate to the mechanism by which they protect. If this is the case, **it may be impossible for us to truly test the efficacy of CCR's formulas in this model.**

We also **completed our comparison of M22 and B2C.** As noted in our report of 2004 results, our concerns over B2C were mostly derived from experiments in which B2C was perfused for 2 hours at 110% of full strength, a protocol that simulates what Alcor often does. When M22 was perfused under the same conditions as B2C, it appeared to give results more similar to but still better than the results obtained with B2C, which reaffirmed our switch from B2C to M22.

B) Whole Body Vitrification

We prepared the majority of a contract research proposal at the request of a wealthy family of Alcor members by the end of 2005 to fund Phase 1 of a three-phase research plan to develop, ultimately, perfected whole body suspended animation. The full plan for Phase 1 was submitted in 2006 and is further described below.
No new research on whole body vitrification was completed in 2005.

C) Kidney Cryopreservation

As proposed, **we tried improving the use of mannitol as an osmolyte** to promote medullary perfusion by raising the mannitol concentration from 100 mM to 150 mM. Very surprisingly, **kidneys treated in this way did not support life** after transplantation. However, using stronger diuresis with mannitol in vivo before nephrectomy, and using a reduced flush pressure of 40 mmHg and a reduced flush

volume of 60 ml to reduce edema, seemed to be beneficial.

In our previous grant letter, we proposed to look more closely at the effects of perfusion pressure to promote renal equilibration. Figure 1A shows that, based on data collected in both 2004 and 2005, **there is a linear increase in urine refractive index with increasing perfusion pressure from 40 mmHg to 70 mmHg**. There is also a trend for the rise to be faster with M-HES than with H7 HES or N-HES. Figure 1B shows the effect of pressure on postoperative mean peak creatinine level with the use of different forms of HES. Although it appeared that 60 mmHg was very damaging to kidneys in the M22B solution series (data not shown) and possibly when N-HES was used, **60 mmHg was relatively innocuous when both M-HES and M22 were used.** As shown in Figure 6A, the latter condition **also appeared sufficient for renal medullary vitrification** (point above the horizontal dashed line). Unfortunately, **injury at 60 mmHg was much higher than in the 40 mmHg B. Braun HES series** (black point) due to elevation of damage even at 40 mmHg when HES forms other than B. Braun HES were used. We will discuss the data represented by the green boxes below.

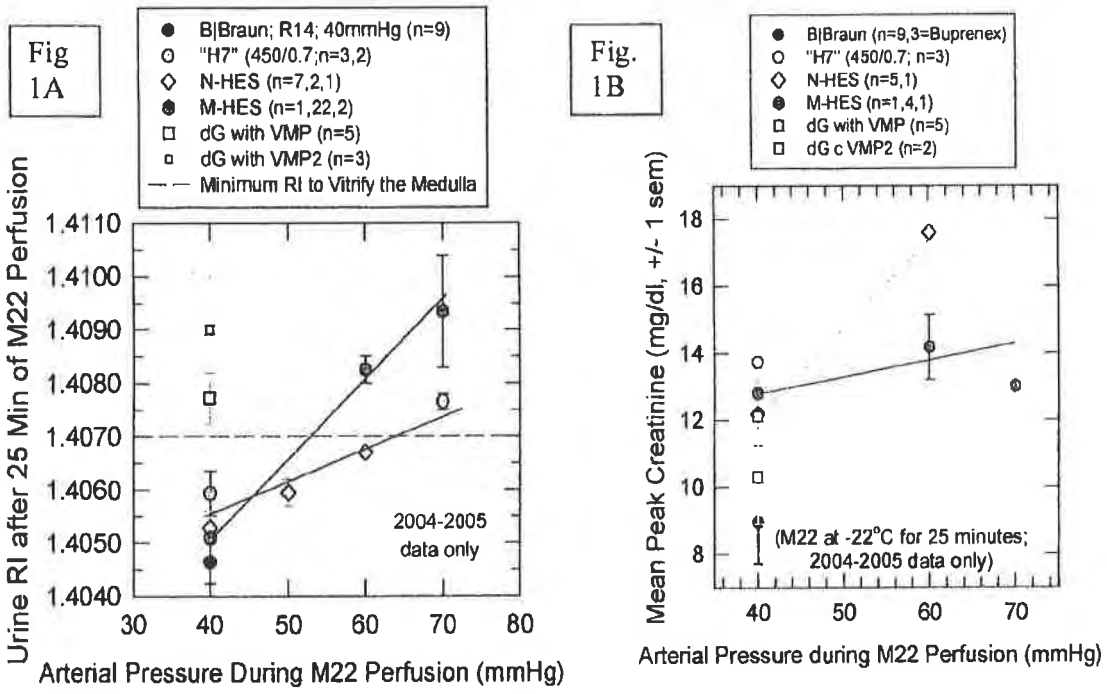

Figure 1: Effect of perfusion pressure on A) renal equilibration with M22 (left) and B) renal injury (right).

The **lack of medullary ice formation at a urine RI value of about 1.408** (achieved using M-HES and 60 mmHg) was confirmed after cooling to -135°C in January of 2005. We therefore made **7 attempts to obtain survival after cooling these kidneys to -100°C. All of these attempts failed, despite appropriate urine RI values in most cases.**

These results are explicable on the basis of the following simple observation. In our original B. Braun M22 series, our peak creatinine level was about 9 mg/dl, and after cooling to -50°C, it rose to about 15 mg/dl, a change of 6 mg/dl. Our baseline peak

creatinine level with physically vitrifiable kidneys and new forms of HES is currently about 14 mg/dl even without cooling below -22°C. If we were to experience only the same rise in peak creatinine level after cooling to -100°C as we previously observed after cooling to -50°C, the peak creatinine level should reach about 20 mg/dl. Unfortunately, this creatinine level is rarely survivable in our current model (no dialysis). If cooling from -50°C to -100°C were to add even another 1 mg/dl to the peak creatinine value, essentially all kidneys would become unable to support life without dialysis. Consequently, **success after cooling to -100°C or below may be unlikely unless the elevated damage associated with the loss of our original supply of HES can be overcome.** Unfortunately, there was no reason to believe that any untested form of HES that might be acquired would be any better than the B. Braun substitutes tested to date.

To determine whether anything was wrong with our basic transplant method or with our perfusion machine, we perfused 5 rabbit kidneys with HES-free carrier solution for 5 hours at 3.5°C, transplanted them, and obtained excellent post-operative creatinine levels. We then repeated the experiment with the inclusion of 2% M-HES. Although no statistically significant difference was obtained, we did find that **there was a trend for M-HES-perfused rabbit kidneys to be more damaged than kidneys that were perfused for 5 hours in the absence of M-HES**, other conditions being similar. This result is consistent with the possibility that M-HES and the other replacement forms of HES tested to date are damaging, and that this toxicity increases in the presence of M22.

We concluded that a radical remedy to the HES problem was required. The remedy we chose to investigate was the replacement of HES with decaglycerol. Although we clearly showed that HES dramatically improves renal equilibration in comparison to no HES, and although decaglycerol is too low in molecular weight to serve as a colloid the way HES does, we postulated that, like mannitol, decaglycerol might be able to act as an osmolyte instead of as a colloid and thereby achieve the same goal of opening up the medullary circulation. Further, although this use of 100mM and especially 150 mM mannitol was found to be possibly toxic to the kidney in our model, we felt this might not pertain to decaglycerol in view of its known extraordinary non-toxicity and its higher molecular weight, which would avoid leakage into the renal cells. Consequently, we replaced 2% HES at the beginning of the perfusion with 2% decaglycerol (dG), and 1% and 2% HES during the washout phase of the perfusion with 2% dG as well. **The results were dramatic.** As shown in Figure 2, using dG **radically increased renal perfusate flow at all stages of introduction and washout of M22 and, as shown in Figure 1, dramatically improved renal equilibration with M22 at 40 mmHg.** Figure 1A indicates that **the use of decaglycerol at 40 mmHg (green boxes) gave equilibration similar to that obtained with M-HES at 60-70 mmHg but with lower peak creatinine levels (Figure 1B, Figure 2B).**

Figure 2B shows our early results with extending the decaglycerol substitution effect further (the VMP2 method, described below). The results were substantially better than our initial dG protocol: **our peak creatinine levels were generally better despite urine RI values approximating 1.409, which is as high as we had ever seen as of that time.**

As shown in Figure 3, **we were clearly able to uncouple the often-observed relationship between increasing urine RI and increasing damage.**

The first dG technique described above used VMP as a transitional solution between no cryoprotectant and M22, but VMP has only 1% decaglycerol (dG). This means that the 2% dG used to improve flow was subsequently reduced to 1% prior to

cooling to -22°C and then increased again after this cooling step. In the VMP2 protocol, we retained 2% dG in the perfusate throughout the loading of the VMP-like VMP2 solution, which increased the tonicity of the solution used just prior to cooling to -22°C (VMP2, for VMP containing 2% dG) to 1.3X from the traditional 1.2X. This did not lead to increased chilling injury upon cooling to -22°C, and therefore opened the door to further shifting the perfusion of the impermeant components of our M22 solution into the VMP-like solution prior to cooling to -22°C. This is desirable because it reduces the

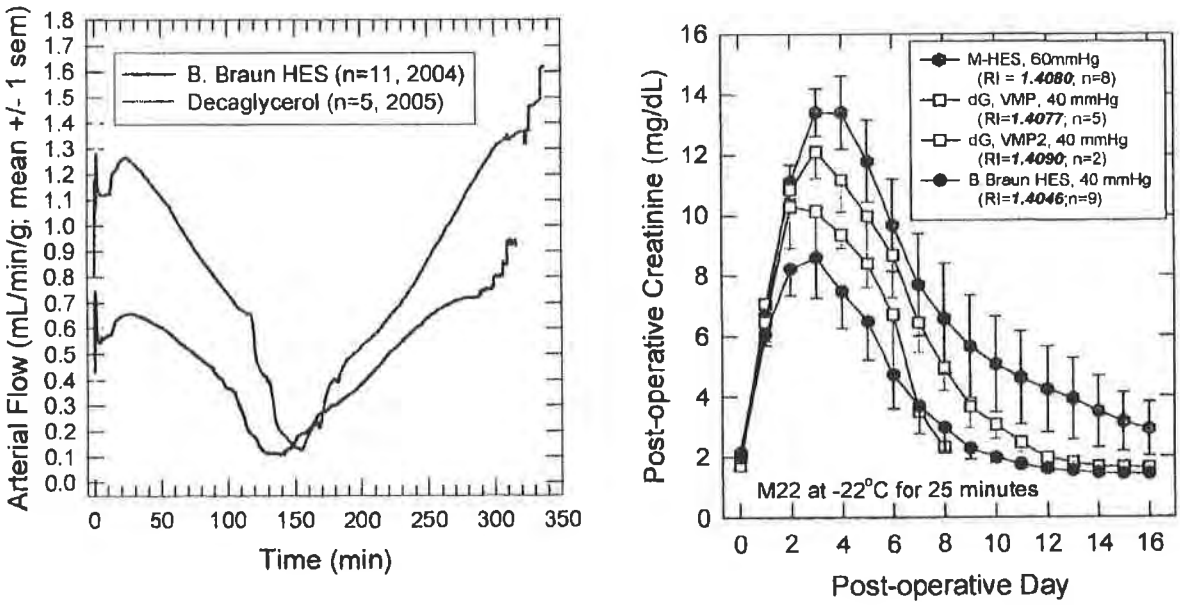

Figure 2: Effect of HES replacement with decaglycerol on renal flow rate (A, left) and renal injury (B, right). Left: renal flow rate during the addition and washout of M22. Data for dG are for the VMP method only. Gray=±1 sem. Right: Comparison between the effects of perfusion with B. Braun HES at 40 mmHg, dG at 40 mmHg, and M-HES at 60 mmHg on post-transplant creatinine levels. Also shown on the right are our first results from a second dG protocol involving the use of VMP2 vs. VMP (n=2).

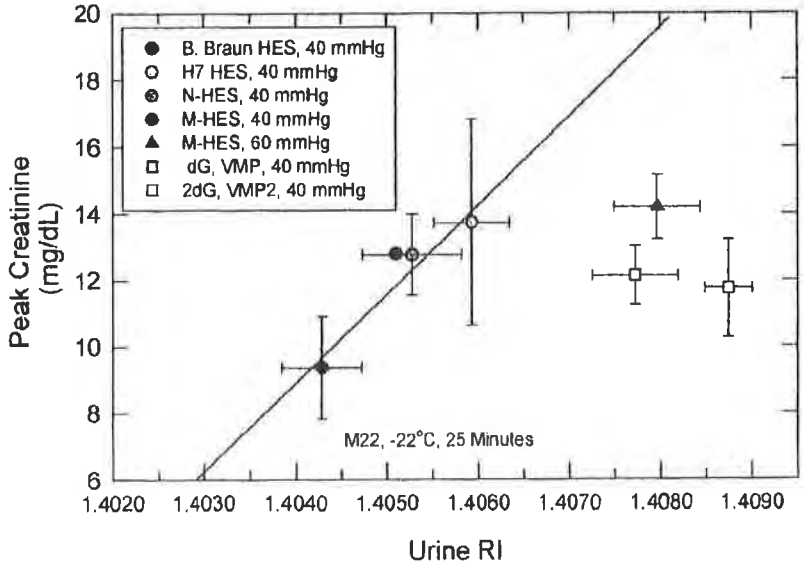

Figure 3 (left): Dissociation of renal injury from renal equilibration using dG substitution for HES (green boxes; for VMP2 method, n=4; previous figures not yet updated).

amount of equilibration required at -22°C, where viscosity is higher and membranes are more rigid, and should also reduce osmotic damage during the onset of M22 washout.

D) Cornea Vitrification

We were able to perform or cause to be performed **transplants of vitrified human corneas into rabbits, monkeys, and human beings.**

To permit these experiments, we first developed, as proposed in our NIH grant and our last grant letter, **a device for the maintenance of corneas near -145°C during transcontinental cornea transportation**. This device, the CorneaPorter, is shown in **Figure 4**. It was created by modifying a commercial "CryoPorter" liquid nitrogen "dry

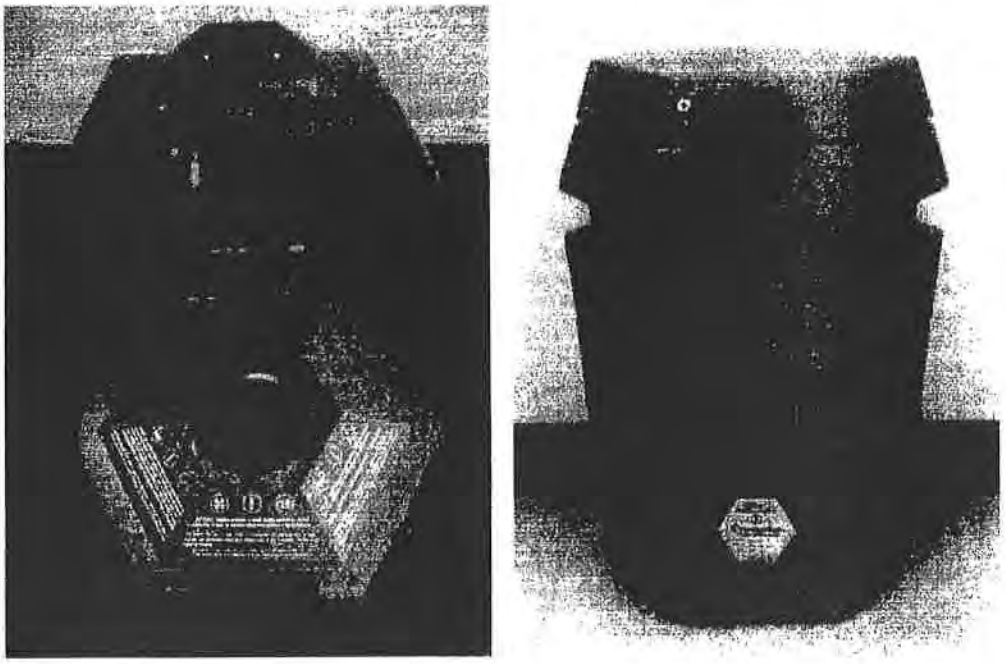

Figure 4: CorneaPorter portable vitrified cornea shipper. Left: As seen from above, with the open lid shown on top and the open mouth of the shipper shown at the bottom. Right: Side view.

shipper" by the addition of an internal "corneastat" of the kind used under non-transport storage conditions in our laboratory. The corneastat uses a battery-powered temperature controller to operate a heater that can maintain temperatures anywhere between -130°C and -190°C for up to four days during shipment in the CryoPorter.

The CorneaPorter was used to ship human corneas one way to China and the island of St. Kitts in the eastern Caribbean, and two ways to Texas and back. The initial prototype used for shipment to China **failed en route**, apparently due to improper orientation during transport and the lack of adequate thermal insulation, arriving at its destination without liquid nitrogen. An improved prototype sent to St. Kitts **survived shipment**, arriving in St. Kitts in a trial run (no corneas) at the desired -145°C. Unfortunately, when it was shipped to St. Kitts a second time bearing one vitrified human cornea destined for

transplantation into a monkey, it **failed to control temperature** but did retain liquid nitrogen, allowing the cornea to arrive near liquid nitrogen temperature. The latter failure seemed to be related to a tendency for batteries to vibrate loose during shipment. We therefore secured the batteries more strongly and shipped more corneas to Dean Barry's wife in Texas, who then shipped them back to 21st Century Medicine. In this test, **the CryoPorter failed again**, this time due to breakage of the heater wires due to impacts en route. **The CryoPorter was further "hardened" by the use of military-grade battery holders and cryogenic-rated wire for the heating system and was successful in all subsequent tests.**

Chinese (Human Clinical) Studies. As noted above, we shipped human corneas to China as part of a clinical pilot study arranged with the Department of Ophthalmology, the First Clinical College, China Medical University No.155, Shenyang, China. Because these corneas were lost in shipment as noted above, we were fortunate to be able to obtain a limited number of local human corneas, vitrify them in China, warm them back up, and transplant them. **Unfortunately, all of the transplanted corneas failed**. **We believe these failures are due to local factors in China that are very different from the conditions prevailing in the United States**, including the lack of baseline clinically used preservative solutions and the resulting inability to schedule surgeries electively. In addition, our first failure was associated with a severe eye infection that may have damaged the vitrified cornea. In addition to opening our eyes about the virtually non-existent nature of eye banking in China, we also learned of several economic and political infrastructure issues that make it difficult to do business in China.

Monkey Studies. One vitrified cornea and two fresh human corneas were shipped to the island of St. Kitts and transplanted into two vervet monkeys by Dr. Wu at the Behavioral Sciences Foundation there. The vitrified cornea arrived at St. Kitts at about -196°C and was rewarmed and washed free of M22 by Dr. Wu. When transplanted into a female recipient, **the vitrified cornea became cloudy, although not opaque as experienced in our human trial in China. After 1-2 weeks in vivo, the appearance of the cornea appeared to improve**, but specular microscopy was not successful in visualizing the endothelium even 7 weeks after transplant although, from one angle, half of this cornea appeared to be clear at 7 weeks post transplant. The two control corneas arrived in St. Kitts partially infiltrated, which made them basically opaque over about 50% of their surfaces. This infiltration had not resolved by 7 weeks after transplantation into a male recipient, but we were able to get good endothelial images at about 1 month post-transplant as well as corneal thickness measurements. **All corneas were clearly alive 5 weeks post-transplant,** and surgical complications or post-operative problems such as rubbing of the eyes, signs of inability to see, rejection, or infection were not seen.

Rabbit Studies. Because of the problems we observed with corneal opacity or cloudiness in the Chinese and St. Kitts trials, we decided to repeat our basic human-to-rabbit model to verify that nothing has changed in our methodology that might be affecting our results. In the course of these studies, we transplanted a cornea that had been **vitrified almost six months** before as well as two corneas that were shipped to Texas and back, having gone to about -190°C in the process, as well as two freshly-vitrified corneas. **Although we saw a little haziness in most corneas on days 1 and 2, all cleared up by day 3-4 except for the cornea preserved for longer than 5 months**, which appeared somewhat cloudy a few days after transplantation but survived. Some of the corneas in these studies were studied by vital staining, cell junction staining, and scanning electron microscopy, and **seemed to be typical of what we had seen in the**

past by these measures. **Storage for 174 days seemed to give results similar to storage for shorter periods of time.**

These studies suggest that although there did seem to be more damage in corneas transplanted in 2005 than in corneas transplanted prior to 2005, the damage was reversible and not reflective of the problems seen in China and St. Kitts. Therefore, **our basic method still seemed to work, and problems outside of our lab must be related largely to local issues rather than to the development of problems with our basic methodology.**

E) Liver Slice Vitrification and Cold Storage

Near the end of 2005, Dr. Fahy, Mr. Barry, and Mr. Horswell met with Dr. **Allison Vickers of Allergan, Inc.,** and a visiting colleague from GUIDE in the Netherlands to discuss liver slice vitrification. This meeting arose from our work with T-cubed, which put us in touch with Allison and Vitron, Inc., in Arizona. Vitron is a small company that does drug screening testing for pharmaceutical companies using human livers. Allison does liver slice experiments for other purposes, but feels the ability to bank liver slices would transform drug testing and have a number of other benefits. The meeting went well, and Allison proposed collaborative studies in 2006.

To prepare for these studies, **we began establishing our basic methodology for liver slices** in November, 2005. At first we were hard-pressed to maintain the viability of even control liver slices. However, we gradually overcame this problem and **established TransSend as a good storage solution for rabbit liver slices**, perhaps even superior to UW solution. We also **found liver slices could tolerate M222 exposure at 0°C without injury**.

F) Cryoprotectant Technology

Efforts were made to find the absolute best combination of cryoprotectants for minimizing toxicity of M22-like solutions in rabbit kidney slices. **A number of solutions were found that were competitive with or perhaps better than M22,** the chief solutions among these being E22 and MEG. However, **M22 appeared to be a bit more stable against ice formation than these solutions**, particularly E22.

Experiments were done to establish the titration curve for neutralization of the toxicity of N-methylacetamide (NMA) by DMSO. NMA was non-toxic up to at least 20% w/v, but combining 20% NMA with 20% DMSO was damaging.

We also ran the titration curve for neutralization of toxicity of **21CM's novel cryoprotectant developed for use by Genzyme.** This cryoprotectant (GA) was found to be limited in solubility to about 27.5% w/v and to have no toxicity up to that limiting concentration. Combining a 20% w/v concentration of GA with 20% w/v DMSO yielded about 97.5% recovery of K/Na ratio. Interestingly, and uniquely, combining 20% GA with 20% ethylene glycol allowed an equally good result to be obtained. This could open another avenue to the development of low toxicity cryoprotectant solutions.

G) Vitrification Physics

Ice Control

Frost-resistance for booster rockets. 21CM was contacted by two different research centers of the National Aeronautics and Space Administration **(NASA)** regarding the possible application of our ice blockers and cryoprotectant solutions in inhibiting ice formation on parts of spacecraft being filled with cryogenic fuels for launch. Although it was unlikely that our ice blockers and solutions as currently formulated would be useful, one reformulation was devised and tested that may be useful. Specifically, a solution of propylene glycol, ice blockers, and surfactant in water was prepared that forms **a vitreous foam** on cryogenic surfaces to protect them from ice formation. This foam will solidify at about –110°C. An ethylene glycol-based foam could go glassy at about -135°C, which might allow less foam to form or might allow formed foam to be more easily stripped from the spacecraft when it is launched. In addition, similar foams containing a mixture of ethanol and methanol might be better able to resist surface frosting by being able to dissolve more water.

Further testing and development would require the execution of a non-disclosure agreement by NASA. Although NASA expressed considerable interest in pursuing some of our ideas, they wanted answers before the shuttle launch, and there was no time to develop practical experience before then. After the launch, interest seemed to shift to other areas, and we have not heard from NASA since.

M22 Stability. Measurements of the critical cooling and warming rates of large volumes of 21CM's most successful vitrification solution to date, M22, were further refined. The results formed the basis of a presentation at the 2005 meeting of the Society for Cryobiology.

Antifreeze protein (AFP) studies begun the previous year in collaboration with **Agrigenesis Biosciences, Ltd.**, of New Zealand were continued. The Agrigensis AFP was of interest because unlike most other AFPs, there is a method to produce the Agrigenesis protein in large quantities if a market is found for it. Two versions of the AFP were studied, a "His"-tagged version and a "Nus"-tagged version. Solution were prepared of 57% w/w EG (ethylene glycol) plus either 0.04% AFP or 0.04% X-1000 ice blocker. X-1000 inhibited devitrification (ice formation during warming from vitrification), but both versions of the protein were found to be ineffective in this model. Addition of 0.004% proteins to 0.04% X-1000 did not improve performance of the X-1000, and no futher studies of Agrigenesis proteins are planned at the present time.

Modelling of Ice-Solute Interactions. The possible ice bonding effectiveness of the potential ice blockers **cyclohexanediol and benzenediol** were studied using Hyperchem software. Molecular dynamics simulation revealed that 1,3-trans-cyclohexanediol and 1,3-benzenediol break off the surface of ice at a temperature of only 70K. However 1,3-cis-cyclohexanediol stays bound for 10 picoseconds at a temperature of 250K. This compares to a binding time of 100 picoseconds for syndiotactic 1,3,5,7-heptanetetrol, the model small molecule used for studying ice inhibition by polyvinyl alcohol, the base compound of the X-1000 ice blocker. 1,3-cis-cyclohexanediol has been promoted as an ice blocker by Organ Recovery Systems, Inc.

Supercooling studies. We **redesigned our supercooling screening apparatus** to eliminate problems of use that we encountered before, including the possibility of interfacial nucleation at the air-liquid interface by airborne ice crystals and problems with thermocouple placement and nucleation by thermocouple wire. The new apparatus was designed to allow us to resume our **search for novel antinucleators** that may be able to complement our existing X1000 and Z1000 products and produce stable and reliable supercooling of macroscopic volumes. No new agents were discovered in 2005, but it is

of interest that our previously-established high-temperature antinucleator, polyglycerol (PGL), was at best only weakly effective in this assay system, and X-1000 was inactive, suggesting that previously-observed high-temperature activity by PGL may have been due in part to prevention of interfacial nucleation.

Fracture Avoidance and ITS Elaborations

Avoidance of Cornea Fracturing. It was found that **vitrified corneas could be submerged under liquid nitrogen and subsequently rewarmed without fracturing** if the following temperature exposure protocol were followed. For cooling, a successful protocol was: -145°C for 8 days followed by -160°C for 1 hour followed by -170°C for 1 hour and -180°C for 1 hour, after which the corneas could be immersed in liquid nitrogen at -196°C overnight. For warming: -180°C for 1 hour, -170°C for 1 hour, -160°C for 1 hour, and then back to -145°C for 2 hours. It not yet known if a more rapid cooling and warming protocol would also have permitted recovery from liquid nitrogen temperature without fracturing.

Large ITS Unit. A design was presented to the Alcor Life Extension Foundation for the construction of an economy ITS unit for the storage of several cephalons and brains within a common thermal environment. Alcor agreed to purchase the unit, and it was tentatively scheduled for shipment to Alcor before the end of 2006.

H) Kidney Cold Storage (TransSend)

Control rabbit kidney transplants were carried out involving 24 hours of storage with Renasol-2 and TransSend-9 because of concerns that our basic transplant baseline may have shifted to higher postoperative creatinine values. We found that, indeed, **we were not able to replicate our original zero-damage baseline** results after 24 hours of 0°C storage. On the other hand, some element of stochastic variation in renal response has been noted in most of our other transplant groups, and the problems seen in 2005 may simply reflect good luck obtained in previous years.

In order to prepare for a re-submission of our Phase 2 NIH grant for the development of TransSend, three control TransSend transplants were done after 24 hours of cold storage, and 8 transplants were done with TS10, which lacks chlorpromazine and contains extra decaglycerol as an osmolyte. The results were not as good with TS10, but were good enough to make TS10 a viable alternative should the FDA object to the use of chlorpromazine in TransSend.

Two additional transplants were done after 24 hour storage in Renasol-10. The results were good and provide additional perspective on the effectiveness of TransSend.

I) Cardiac Cold Storage and Intermittent Perfusion

The research for this project is being paid for by a phase 2 SBIR grant from NIH, which covers dog heart transplants at the University of Rochester (UR) and human heart perfusions with human blood at 21CM. The object is to show that dog and human hearts survive 24 hours of ice storage with University of Rochester Solution (URS) using transplantation with life support (at UR) or function during blood perfusion (at 21CM) as end-points.

The **Human Organ Lab (HOL)** at 21CM was successfully completed, and **new office space** to house staff displaced by the HOL was completed as well. Numerous components for the blood perfusion machine that will be used to evaluate preserved human hearts at 37°C as well as necessary new equipment such as a blood centrifuge and a clinical defibrillator were purchased. The **perfusion machine components were largely assembled** (early stages shown as of June 1st, 2005 in Figure 5), and the largest uncompleted tasks for this machine were in software development and computer interfacing with the machine components as of the end of 2005.

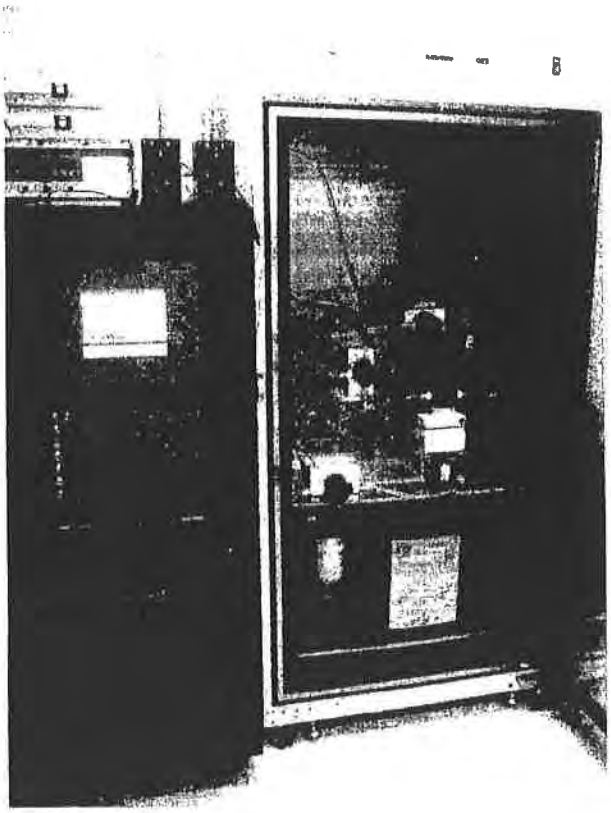

Figure 5: Beginning of the construction of the human heart perfusion apparatus. Computers and monitors on the left, 37°C cabinet with components partly assembled on the right. On the bottom of the cabinet is the blood reservoir (left) and the blood pump (right). On the top shelf of the cabinet are high-volume solenoid valves, a lift-table to create varying preloads, and the organ chamber (scarcely visible at the middle of the shelf).

Figure 6 (left): Engineering model of thermoelectric heat extraction from an ice chest. The thermoelectric device (TED) is shown penetrating the right wall of a test styrofoam ice chest; the laptop running the TED and monitoring its results is shown at right.

In addition, design work and initial experiments were done on the installation of a **thermoelectric cooling system in the original intermittent perfusion device** developed in Phase 1 of this grant (the CardioStat) for automating the extended preservation of dog and human hearts, in keeping with mandates of the Phase 2 grant. An initial test thermoelectric device was purchased and tested on a model styrofoam box (Figure 6). Based on the performance of this unit, a larger unit that should be equal to the job of freezing the water in the CardioStat was ordered for testing.

As required by the grant, **UR personnel** performed a series of **dog heart transplantation experiments** intended ultimately to demonstrate the viability of the canine heart after 24 hours of cold storage with URS based on life support by the heart after transplantation. Their experience is summarized in the following table. Because of the problems they experienced, we were informed near the end of 2005 that the experiments had been put on hold by the UR animal committee. This forced us to alter our own research plans for 2006, as described below.

Month(s)	Experiment (No. done)	Results
Feb-April	Practice Autografts (5)	Failure: bleeding, inadequate bypass pressure
May	Practice bypass (CPB) (1)	Success: but anemia post-operatively
June	Tx after 24-hr storage (1)	Failure: no resumption of beating
August	Allograft, no storage (1)	Failure: bleeding from anastomosis, failure to anticoagulate successfully
August	Allograft, no storage (1)	Failure: surgical wound to the aorta
September	Tx after 24-hr storage (1)	Failure: inadequate bypass pressure, hypothermia, asphyxiation; no bleeding
September	Tx after 24-hr storage (1)	Failure: surgeon not present for transplant
October	Tx after 24-hr storage (1)	Failure: diseased heart, protamine effect (?)
November	Tx after 24-hr storage (1)	Failure: bleeding, unchecked anticoagulation, inadequate systemic pressure, edema

J) Hepatocyte Cold Storage

TransSend and a **variant of TransSend called TS-HC** for "TransSend-Hepatocytes" were tested by Tissue Transformation Technologies (TTT, or T-Cubed) for **efficacy in preserving isolated hepatocytes for 24 and 48 hours of cold storage**. Our solutions were compared to a competing solution, Hypothermosol-FRS, which has been licensed to TTT for use by Bio-Life Solutions, Inc. **TransSend beat Hypothermosol-FRS** (Hypothermosol plus free radical scavengers) after 24 hours of hepatocyte cold storage, but not after 48 hours of cold storage. Unfortunately, TTT went out of business before additional testing and additional variants could be examined.

K) Contract Research

Genzyme. We completed additional studies for our Genzyme contract in 2005 that gave critical information on the role of VeGD exposure time and the cooling and warming protocols that gave the best results. We also assembled all data so far into four graphs that plot results as a function of cooling rate or warming rate when the data are normalized to either controls or VeGD treated slices that were not vitrified.

These results were presented to Genzyme and were then applied in-house at Genzyme to porcine cartilage. John Phan, who did the kidney slice trials at 21CM in preparation for these experiments, **visited Genzyme** to assist the team there with the process of adding cryoprotectant, vitrifying the cartilage, rewarming the cartilage, and washing out the cryoprotectant. The Genzyme team then digested the cartilage into isolated cells, plated them, and scored the results. Although there was a delay in adhesion of the liberated chondrocytes to the plastic culture dishes, ultimately the growth of the cells was identically equal to that of untreated porcine chondrocytes. Therefore, **the vitrified samples behaved almost as though they had never been treated at all.**

After John left, Genzyme tried the experiment for the first time on human cartilage samples. One of these samples behaved like the porcine samples, recovering to about **90% of untreated human control performance**. The remaining samples fell short, probably because **the Genzyme group deviated from the procedure** established for porcine cartilage by packing too much cartilage into too little cryoprotectant, which would dilute the cryoprotectant and therefore prevent successful vitrification. This was pointed out to Genzyme, but nevertheless, this was the end of their experiments to date as far as we are aware. We are told that the lab that was doing these tests has been redirected to other activities and that there **may no longer be interest in this project** even though it is essential for the profitability of Genzyme's commercial Carticel^{R} product, a rather perplexing development and a strange ending to Genzyme's $45,000 investment in 21CM research to date.

Despite Genzyme's lack of follow-through, a successful process for cryopreserving cartilage **would have considerable commercial value**. Given that the successes obtained were obtained using a novel preservation solution containing a novel cryoprotectant, none of which is covered by existing 21CM patents, 21CM is planning to patent the solution and the process and seek other interested partners who can take our technology commercial.

Agrigenesis. We studied novel **antifreeze proteins from Agrigenesis** (now Genesis) that are derived from grass, with negative results as reported above. These studies were severely handicapped by the lack of adequate protein, and if repeated with 0.04% protein, for example, might give different results. Therefore, these experiments do not rule out further studies on antifreeze proteins in general.

L) Collaborative Cryopreservation Research

Dr. Duman of Notre Dame University sent us **hemolymph from the Alaskan beetle Cucujus** after concentrating it up to 4 or 5 fold to simulate the dehydration of the insects that takes place as temperatures fall in the winter. The 4 x concentrate was found to vitrify at any cooling rate higher than 5°C/minute, and at 5°C/min froze with an energy of 3.76 J/g at -55°C (only 1% ice formation). When the sample was cooled quickly to vitrify it and then warmed at different rates, it devitrified unless warmed at 160°C/min or faster. The thermodynamic melting point of the sample was -32°C, and the glass transition temperature was -98°C. Based on these results, these beetles will escape freezing in the winter only if they are concentrated to the 5 x level, 4 x being insufficient. If they make it to 5 x, they would never vitrify because the environmental temperature would never reach -98°C, but they would never freeze either, **and would instead pass the winter in a supercooled state** not far below their thermodynamic melting points.

Inge de Graff of the **Groningen University Institute for Drug Exploration (GUIDE)** in the Netherlands did additional work with vitrification solutions supplied by us, mostly dealing somewhat unsuccessfully with the issue of chilling injury in rat liver slices. However, she submitted her previous work with our solutions to Cryobiology for publication in 2005.

We were contacted by **VetStem**, a California company (www.vetstem.com) that wanted us to vitrify horse umbilical cord stem cells for possible therapeutic applications. After due consideration, we decided, in 2006, not to pursue this research due to an inadequate anticipated return on our expected investment of time and resources.

2. Personnel and facility changes

In November of 2005, we began construction of the **Human Organ Lab** and new office space for the people displaced by this new lab. The construction was completed in March of 2006.

In 2005-2006, **laboratory renovations required for the whole body vitrification** project were designed, and a final version was designed in July of 2006. Construction began in late September, 2006, on a new whole body vitrification laboratory and an adjacent fabrication shop. The estimated date of completion for these two rooms is December of 2006.

In July of 2006, our surgical assistant, **David Ta**, departed 21CM and was replaced by Lenetta **Griffin and Laura Chang**.

We were advised in September, 2006 that our transplant surgeon, **Dr. Jun Wu**, had made a decision to leave 21CM and devote himself to other pursuits. Given his expected departure during January, 2007 (exactly 7 years since his hiring), a search for his replacement is underway. Advertisements have been placed on Monster.com., on Medzilla.com., and networking through veterinarians and recent research institution contacts is beginning as this Letter is being written. A general search for other technical positions to support the whole body project has simultaneously begun using the same initial strategies.

3. Publications

2005 Scientific Publications

Dhahbi, J.M., Mote, P.L., Fahy, G.M., and Spindler, S.R. Identification of potential caloric restriction mimetics by microarray profiling. Physiol. Genomics 23: 343-350, 2005.

Fahy, G.M. Cryopreservation of complex systems: the missing link in the regenerative medicine supply chain. Rejuvenation Research 8 (Supplement 1): S-30, 2005.

Fahy, G.M. Vitrification as an approach to cryopreservation: general perspectives. Cryobiology 51: 348-349, 2005.

Wowk, B. Anomalous high activity of a subfraction of polyvinyl alcohol ice blocker, Cryobiology 50, 325-331, 2005.

Wowk, B., and Fahy, G.M. Toward large organ vitrification: extremely low critical cooling and warming rates of M22 vitrification solution. Cryobiology 51:362 , 2005.

Wowk, B. Controlled temperature environments for maintenance and shipment of vitrified tissue. Cryobiology 51: 382, 2005.

Hagedorn, M., Pan, R., Cox, E.F., Hollingsworth, L., Krupp, D., Lewis, T.D., Leong, J.C., Mazur, P., Rall, W.F., MacFarlane, D.R., Fahy, G., and Kleinhans, F.W. Coral conservation and cryopreservation. Cryobiology 51: 352, 2005.

2006 Scientific Publications

Hagedorn, M., Pan, R., Cox, E., Hollingsworth, L., Krupp, D., Lewis, T.D., Leong, J., Mazur, P., Rall, W., MacFarlane, D., Fahy, G., and Kleinhaus, F.W. Coral larvae conservation: Physiology and reproduction. *Cryobiology* 52: 33-47, 2006.

Pichugin, Y., Fahy, G.M., and Morin, R.M. Cryopreservation of rat hippocampal slices by vitrification. ***Cryobiology*** 52: 228-240, 2006.

Fahy, G.M., Wowk, B., and Wu, J. Cryopreservation of complex systems: the missing link in the regenerative medicine supply chain. *Rejuvenation Research* 9: 279-291, 2006).

Tan, Y., Hori, N., and Carpenter, D.O. Electrophysiological effects of three groups of glutamate metabotropic receptors in rat piriform cortex. Cell Mol. Neurobiol. Aug 2, 2006 (Epub ahead of print).

de Graaf, I.A.M., Draaisma, A.L., Schoeman, O., Fahy, G.M., Groothuis, M.M., and Koster, H.J. Cryopreservation of rat precision-cut liver and kidney slices by rapid freezing and vitrification. *Cryobiology* (in press, 2006).

Fahy, G.M., Wowk, B., and Wu, J. The first surviving vitrified vital mammalian organ. (in revision for resubmission to Cryobiology in 2007).

Lay Publications

Wowk, B. Is Hydrogen Sulfide the Secret to Suspended Animation? Cryonics 26(4): 20, 2005.

Wowk, B. Science of Cold Heats Up. "What We Know Now," September 27, 2005 ([illegible]).

Fahy, G.M.. Advances in Cryopreservation. Cryonics 27(2): 7-8, 2006.

Fahy, G.M. Understanding Ice Damage. Cryonics 27(3): 16-17, 2006.

4. **Scientific presentations and media coverage**

Presentations (2005)

Dr. Fahy delivered an **invited plenary lecture** at the 2005 meeting of the **Society for Cryobiology** in Minneapolis, Minnesota, in July, entitled "Vitrification as an Approach to Cryopreservation: General Perspectives".

Dr. Fahy presented an **invited talk at SENS-2** in September, 2005, in Cambridge in the UK) entitled "Cryopreservation: The Missing Link in the Regenerative Medicine Supply Chain."

Dr. Wowk presented **two oral talks** ") at the 2005 **Society for Cryobiology** meeting in Minneapolis, MN, in July, entitled "Toward large organ vitrification: extremely low critical cooling and warming rates of M22 vitrification solution" and "Controlled temperature environments for maintenance and shipment of vitrified tissue".

Dr. Wowk gave **an invited talk** at the **Eris Society** meeting in August, 2005, in Aspen, Colorado entitled "Suspended Animation: Not Just for Cells Anymore."

Dr. Wowk gave **an invited talk** at the **Immortality Institute Life Extension Conference** in November, 2005 in Atlanta, Georgia entitled "Suspended Animation by Vitrification."

Dr. Wowk gave **an invited presentation** at the **Freedom Summit** in Phoenix, AZ, in November, 2005 entitled "Secrets of Suspended Animation."

Presentations (2006)

Dr. Fahy delivered an **invited presentation** in Bologna, Italy at the **2nd International Symposium on Cryopreservation of the Human Oocyte** on October 5th, 2006, entitled "Beyond Empiricism in Cryobiological Research"

Dr. Fahy presented an **invited featured talk** at the 2006 **Alcor** meeting in Phoenix, Arizona on October 8th, 2006, entitled "Research Toward Suspended Animation".

Dr. Wowk presented an **invited talk** at the 2006 **Alcor** meeting in Phoenix, AZ on October 8th, 2006 entitled "The Cryobiological Basis of Cryonics".

Media Coverage

21st Century Medicine was filmed by the British company, ZigZag, under contract with the **National Geographic Channel** to create a documentary on cryonics that included the cryobiology background necessary to give the show perspective. The program aired in the United States on the National Geographic Channel in September, 2006 and was entitled **"Freeze Me!"** The producer was Virginia Quinn. 21CM was portrayed as a cutting edge research company not connected to cryonics.

We were approached later in 2006 by two film companies from Canada interested in **including 21CM in documentaries on aging.** As of this writing, no firm plans have been made to participate in these programs.

5. Patent Applications and Awards

Applications

PCT Application 60/245959, filed June 17th, 2005. "Extended Organ Preservation," G.M. Fahy and T. Wang. This application is a foreign submission of a previously-corrected US application for **heart preservation with University of Rochester Solution and for a device for carrying out intermittent perfusion.**

A Request for Continued Examination was submitted on May 24th, 2006 and accepted by the US Patent and Trademark Office for U.S. Patent Application No. 09/916,032, "Hypertonic Reduction of Chilling Injury" (G.M. Fahy), now in its final stages of prosecution.

Patents filed for the **use of decaglycerol or lactose to prevent warm ischemic changes** and for protection for LM5 and advanced cryoprotectant solutions in Canada will be documented in our 2007 Grant Letter.

Awards

U.S. Patent 6,869,757 B2, March 22, 2005. "Advantageous carrier solution for vitrifiable concentrations of cryoprotectants, and compatible cryoprotectant mixtures" G.M. Fahy. This is a patent for our **LM5 carrier solution** and newer, **particularly effective cryoprotectant solutions.**

U.S. Patent No. 6,949,335 B2, September 27, 2005. "Polyglycerol and lactose compositions for the protection of living systems from states of reduced metabolism." G.M. Fahy and J. Wu. This is a broad patent for our **TransSend** family of conventional (0°C) organ storage solutions.

German Patent No. DE 699 29 071 T2, August 17, 2006. "Improved Cryoprotectant Solutions." G. M. Fahy and B. Wowk. This is the counterpart to our existing US patent on the **V_{EG} family of cryoprotectant solutions. The same patent has also issued in The Netherlands, Italy, France, and the United Kingdom and is pending in India and Canada.**

U.S. patent application 10/864,921, filed 06/09/2004, letter of allowance received 10/2/06. "Cryogenic Storage System," Brian Wowk and Michael Iarocci (fee for issuance due 12/06/2006). This is our patent on devices for **intermediate temperature storage** of biological samples.

6. Grant Applications and Awards

Grants Submitted in 2005:

A Phase 1 SBIR application to NHLBI entitled "**Resuscitation of Non-Heart-Beating Donor Hearts**," G. Fahy, P.I., for a requested $199,000, was resubmitted on April 1st, 2005. We received word in 2006 that this application, submitted at the suggestion of our collaborator, Dr. Ting Wang, was **not funded** largely because NIH did not believe sufficient hearts would be eligible to be salvaged by our proposed method. We decided not to resubmit the grant for the same reason.

A Phase 1 SBIR application to NIMN entitled "**High throughput optical recording system**," Y. Tan, P.I., for a requested $159,000, was submitted on December 1st, 2005. This work was to have been in collaboration with Dr. Wu in Georgetown University, an expert in this field. Unfortunately, this grant was not funded due to technical questions raised by some of the reviewers. This grant **will be re-submitted** either as an independent grant or as part of a Phase II application for our Phase I SBIR, "Neural Tissue Vitrification," documented below.

A Phase II SBIR submission to NIH entitled "**Improved Renal Preservation**," G.M. Fahy, P.I., for a requested **$1,096,656**, from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), was submitted on December 1st, 2005. Unfortunately, it was not funded due to confusion on the part of the major reviewer and **will be resubmitted** with additional data that should bring enlightenment to the reviewer.

A request to extend our Phase 1 SBIR grant on cornea vitrification for one year was granted.

A request to extend our Phase 2 SBIR grant on "Extended Cardiac Preservation" for one year was granted.

A grant application to the California Institute of Regenerative Medicine is planned involving a three-way collaboration between 21CM, Children's Hospital and Oakland Research Institute (CHORI) and Advanced Cell Technology by the end of 2006. This would fund the demonstration of efficacy of 21CM cryopreservation media for the preservation of embryonic progenitor cells potentially useful for the treatment of many maladies of aging and other conditions.

Awards

A Phase 1 SBIR application to NIMN entitled "**Neural Tissue Vitrification**," Y. Tan, P.I., for a requested **$190,950**, was submitted on 12/02/2005. The final grant award was for $184,915, and the grant period is from 5/1/2006 through 4/30/2007. The grant is aimed at working through experimental variables to arrive at the demonstration of successful cryopreservation of neural tissue by vitrification. The results should be useful for demonstrating the practical utility of cryopreserved brain slices as research tools.

7. Research contracts and licensing agreements

We were awarded and successfully completed a contract with **Genzyme Biosurgery** for **$15,000** for Phase 3 of a project to develop optimal methods for the use of our previously-developed formamide free vitrification solution in 2005.

A **$25,000/year** contract for the licensing of M22 and procedures for its use on whole body patients was submitted to **Alcor** and completed in 2005. This contract is ongoing into 2006 and beyond.

A licensing agreement for the use of a variety of 21CM solutions was signed with **Suspended Animation, Inc.**, in 2006. This has resulted in **$5,000** in income to 21CM so far in 2006.

We were particularly pleased to complete a material testing agreement with Roger Gosden's **Center for Reproductive Medicine & Infertility, Weill Medical College, Cornell University** in September of 2006. This center is reputed to have the highest success rates for all aspects of reproductive medicine in the world. The collaboration will cover human ova, human stem cells, animal ova and ovarian tissue, and possibly human embryos. No payments are involved with this agreement, which is intended to generate revenues through future licensing.

We were also pleased to complete a research agreement in 2006 with **Tecnobios Procreazione**, a fertility clinic in Bologna, Italy, where embryo freezing is illegal and ova cryopreservation is a practical necessity for the survival of the IVF industry. This research will develop novel vitrification methods and solutions for human oocytes. 21CM will have the right to commercialize the technology outside of Italy, and the Italians will have the right to license the technology from 21CM for use in Italy only. This group is working with **Cook Women's Health**, which is a part of a **multi-billion-dollar multinational company** that has expressed a desire to sell 21CM freezing solutions around the world in kit form to facilitate human egg cell freezing. However, talks about the latter possibility are presently little developed, and **Cook is based in Australia, where 21CM has no patent protection.**

A research contract with **the University of Arizona** was also pending at the time of this writing. This agreement will form a three-way collaboration between the Gosden group in New York, John McGrath's group at the University of Arizona, and 21st Century Medicine to pursue the projects mentioned above in connection with Gosden's group.

8. Preliminary results and proposed research for 2006

A) Brain Vitrification

Brain Slice Cryopreservation

This NIH-supported project is well ahead of schedule. On January 3rd, we completed upgrades allowing our cryoprotectant introduction and removal process to be fully automated using Linkable Instrument Network software. We also installed a new programmable freezer in the Neurophysiology Lab with the help of Dr. Wowk.

Part of the grant was to compare three of our standard cryoprotectant formulas, V_{EG}, VM3, and M222, as vitrification media for rabbit hippocampal slices. The detailed results to date are summarized in the table below. "% Recovery" indicates the peak height of the field voltage response to electrical stimulation divided by the peak height

obtained from untreated control slices in the same experiment, using only responding slices to calculate the percent recovery of electrical response. "% Responders" refers to the number of slices that were responsive to electrical stimulation divided by the total number of slices tested (for control slices, this number would normally be in the vicinity of 90%.) "+/- (n)" refers to the results obtained after addition and washout of the stated cryoprotectant only and to the number of slices tested; "v/r (n)" refers to the results obtained after vitrification and rewarming of the slices and to the number of slices tested. The percentage of responders is absolute and is not normalized to the percentage of control slices responding. "K/Na" refers to the potassium: sodium ratio after the stated treatment and rewarming to 37°C for testing as per the measurement of electrical responses.

Performance of Hippocampal Slices after Cryoprotectant Treatment and Vitrification

CPA	% Recovery after +/- (n)	% Responders after +/- (n)	% Recov. after v/r (n)	% Resp. after v/r (n)	K/Na, +/- (n)	K/Na, v/r (n)
V_{EG}	99.5 (16)	82.9 (16)	74.0 (16)	69.45 (16)	1.0 (17)	.88(18)
VM3	90.7 (33)	73.0 (33)	69.9 (30)	68.9 (30)	1.1 (37)	.91(37)
M222	88.5 (19)	81.1 (19)	71.3 (19)	66.5 (19)	.97 (21)	.80(22)

In general, the results were similar for all three cryoprotectants. Statistical comparisons have not been performed as of this writing, but will be available by the end of 2006 as these experiments are completed. There may be reduced electrical activity in the VM3 group, which may reflect the 1.7X tonicity of this group, considering the significant responsiveness of electrical activity to hypertonicity. There may also be more injury from M222 than from V_{EG} prior to vitrification, which would be consistent with the higher concentration of M222, but any such gap becomes narrowed after vitrification and rewarming, perhaps because of the greater stability of M222 during those steps.

The slice team has been working on LTP testing as a measure of biological memory formation in these slices, but had not fully overcome technical problems in making these measurements by the time of this writing. Results are anticipated, however, by the end of 2006.

Figure 7 below shows typical cooling and warming curves during vitrification and rewarming (left) and a typical EPSP recording from a vitrified/rewarmed brain slice in

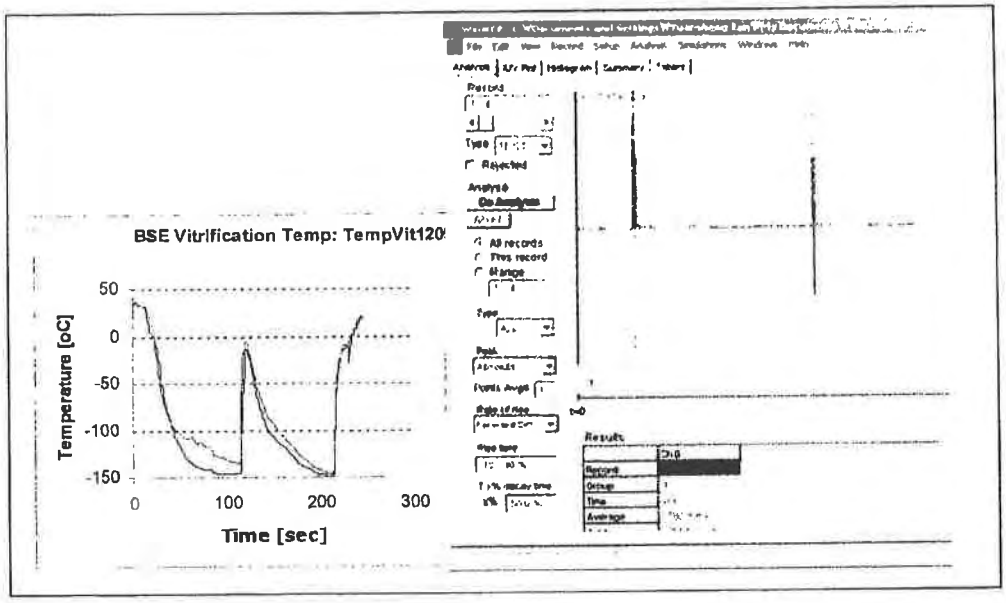

Figure 7 (above): Typical cooling and warming protocol (left) and post-vitrification electrical response (right).
response to a paired-pulse stimulus (right).

Whole Brain Cryopreservation and Cold Storage

A major bottleneck in our research has been the need to convert vast numbers of individual electron micrographs into an organized form so they can be analyzed, compared, presented, and published. Another major bottleneck has been getting tissue samples processed for microscopy and photography. Consequently, we devoted several months in 2006 to **eliminating our backlog** in these areas. We now have many experimental results scanned into our company server and available for viewing and analysis, and a relatively short list of experiments that still need to be photographed. We will complete the preliminary analysis of all of these data by about the end of 2006 and present the results in our grant letter for 2007.

The most important development in 2006 may be the creation of **a new method for getting cryoprotectants to penetrate the blood-brain barrier**. In our model, the control rabbit brain has been found to weigh about 10 grams. After perfusion with M22 by our standard methods, the brain weight drops to under 5 grams, and upon examination the brain is found to be very shrunken on a cellular level. This extreme shrinkage does not cause the structural destruction caused by ice formation, but might lead to the same kind of dehydration injury seen with slow freezing, an undesirable effect, and subsequent removal of the cryoprotectant has so far led to unacceptable ultrastructural injury, perhaps for this reason. Our attempts to avoid this shrinkage effect by chemically and osmotically opening the blood-brain barrier (BBB) were successful in preventing brain weight changes, but still led to too much ultrastructural damage to the brain, and similar problems were seen when we attempted to facilitate permeation by raising the perfusion temperature to +10°C.

But there is another way to consider this problem, and that is by **matching the time course of the experiment to the time course of cryoprotectant uptake by the brain**. All biological systems perfused with penetrating agents tend to undergo a **"shrink-swell" curve,** in which water is initially osmotically withdrawn and cryoprotectant then goes into the cells. In most cases, the withdrawal of water overlaps significantly with the uptake of cryoprotectant, the latter requiring only minutes to an hour to become completed or nearly completed. The question we asked was, is the failure of the brain to take up cryoprotectant the result of absolute impermeability, or is it the result of a "shrink-swell" curve that is shifted to times that are so much longer than the time course of a typical experiment as to make the uptake part of the curve basically invisible? Stated differently, the question is: **what if we just allow more time for the cryoprotectant to go in**? Will it?

The answer at the moment seems to be that it will. However, not all agents penetrate equally rapidly. **Three molar glycerol apparently permeates the rabbit brain fully in six hours or less**, but 3M M22 (minus the impermeant polymers) only permeates the brain partially, final brain weight approximating six grams rather than the 9-10 grams seen with glycerol. Comparing glycerol to ethylene glycol, glycerol appears to penetrate more rapidly, and ethylene glycol appears to be comparable to the permeant mixture found in M22. These interpretations are based on the assumption that weight changes are not secondary to greater vascular damage with glycerol, a point that should be clarified by the ultrastructural results.

The ability to get glycerol into the brain opens the possibility of removing it as well without osmotic injury, an important principle to establish if possible. Hence, we have done experiments in which **1.5M glycerol was introduced over 3 hours and then washed out over 3 hours** so the brain could be fixed and examined for normalcy. The results should be available before the end of the year.

The concept of slow cryoprotectant loading is compatible with another concept we have been developing, which is **the need to keep the brain in viable condition for up to 24 hours before cryopreservation takes place**. It occurred to us that if the body is being continuously perfused for up to 24 hours before cryopreservation, most of this time can be spent loading in at least moderate concentrations of cryoprotectant. Halasz showed, many years ago, that kidneys perfused with moderate concentrations of cryoprotectant for 24 hours were actually more viable when transplanted than those that weren't. **There is essentially no information on the problem of how to maintain brains in viable condition for 24 hours outside the body** (if one excludes Suda's expedient of freezing them at -20°C!), but it is essentially certain that continuous or intermittent perfusion will be needed so as to be able to maintain brain metabolism. Given this baseline condition, the continuous or intermittent introduction of moderate concentrations of cryoprotectants should be feasible. In the case of M22 permeating solutes, if the solutes enter the brain at the rate of 1 molar per six hours (a reasonable preliminary inference from the shrinkage data), then the brain can be loaded with up to 4M M22 in 24 hours with no shrinkage at all. This concentration may be acceptable, and stepping to the final 9.3M complete M22 solution should then reduce brain mass to no less than about 8 grams, which should be mild enough to be non-damaging, assuming a non-osmotic brain mass of 4 grams.

This approach could make it difficult to check brain viability because nominally the washout time should be 24 hours long as well. However, by adjusting the composition of the cryoprotectant solution, using simultaneous changes in carrier

tonicity, and using the maximum acceptable cryoprotectant concentration gradient across the brain, both the addition and washout steps might be amenable to acceleration. Further, mild BBB opening coupled with slow CPA introduction and removal may give much better results than BBB opening followed by the comparatively rapid changes in CPA concentration we have used heretofore. Finally, we can short-cut the process by removing the brain and slicing it to allow more rapid washout of cryoprotectants.

To date **we have completed a number of control experiments in which the brain is perfused for 24 hours at 10°C** and then fixed to determine whether its ultrastructure has been preserved. Such preparations can be tested for viability using a variety of methods should their structural preservation be intact enough to justify such testing. Our initial 10°C perfusions have not utilized perfusate oxygenation, the instillation of a hydrogen sulfide generating system, or alternative temperatures such as 15°C or even 20°C, but all of these modalities can be introduced if necessary or desirable to allow 24 hour preservation, which in a cryonics context is necessary for transport much of the time. If necessary, we will modify our current brain perfusion machine to enable it to maintain and document active brain metabolism for up to 24 hours.

Although we do not have the results of these 10°C perfusions yet, **24-hour static cold storage experiments at 0°C** show that gross histology is still present with most storage methods, and in at least some areas may be adequate. In view of highly variable ultrastructural results from region to region, we followed up our earlier cold storage results in 2006, this time using **elevated perfusion pressures for fixation to ensure that the fixative reaches all areas of the brain more uniformly**, and are awaiting the results of this more definitive protocol.

These lines of research will be pursued further in 2006 and developed still more in 2007 if they appear to be beneficial. In addition, if necessary we will consider **simultaneous perfusion of the brain through the vascular system and the cerebrospinal fluid pathway**, which directly bathes the brain surface, in order to speed up equilibration.

B) Whole Body Vitrification

At the end of January, 2006, a major research proposal was submitted to a family valued at well over $1 billion that had requested us to draft a plan for vitrifying whole bodies. They elected not to fund the research, but LEF decided to move forward with the project as proposed. **The proposal involves determination of the best way to vitrify the body and the development of non-invasive measurements of perfusion adequacy.** Many features of the proposal **require the creation of new technologies**, including technologies for visualizing ice in vitrified bodies, an ability that would be of great value in the study of the vitrification of isolated organs and brains as well.

In preparation for beginning these studies, plans were drawn up for creating a new laboratory for whole body vitrification research and that would allow us to accommodate the extra staff members required for this project, and construction was begun. The suppliers of whole body cryosectioning equipment (Leica in Germany and Hacker in the US) were again contacted about the engineering requirements to adapt their systems to our needs, but ultimately those discussions broke down as **neither company seems able or willing to make the changes needed to section vitreous rabbits near -130°C**. Our current plan is therefore to **mill the rabbits** rather than to section them, using a milling machine similar to that used for **the Visible Human Project** in which frozen human

cadavers are photographed in a series of thin layers to provide an atlas of human anatomy. In our case, it will be necessary to control the interface temperature adequately to avoid unacceptable heating, something that may be accomplished using thermographic imaging of the surface to record and thereby control local temperatures.

Cutting glass is difficult because it is brittle and may tend to chip or melt, but the experience of Dr. Fahy in cryosectioning vitrified kidney slices is that a) when the temperature is too low, the sections crumble into fine powder, which in our application can simply be brushed away to reveal the surface beneath, and b) there seems to be a narrow temperature window of about plus or minus 0.5°C within which vitreous sectioning can be done successfully. Either of these two temperature regimes should be acceptable for our study, and establishing a low temperature milling machine should be far easier technically than creating a machine that can generate thin cross-sections through vitreous rabbits.

Further infrastructure required for this project is being assembled and designed. It is anticipated that the better part of a year will be needed to design and build **the whole body perfusion machine**, to select and install **a scanning electron microscope**, and to build other equipment needed for the project, such as a controlled warming device that will allow tissue samples cut out of rabbit cut surfaces to be watched as they rewarm to allow us to study devitrification in situ. Therefore it is expected that most of 2007 will be devoted to establishing the basic capability to do these experiments, and that the hiring of most new staff to carry out the necessary experiments is not likely until near the end of 2007.

Additional insight into whole body vitrification was obtained by additional analysis of our previous data, as shown in Figure 8. This figure shows **observed (points) and expected (smooth line) amounts of ice formation in rabbit tissues** based on the two rabbits we perfused with M22 in 2003; the amount of ice is derived from the heats of melting in different tissues and the expected amount of ice is derived from the melting point (Tm) of each tissue sample. At the right is a key indicating the relationship between the amount of heat required to melt the sample and the fraction of the mass of the sample that was converted into ice before the ice was melted.

The new feature added to this graph is the solid line providing a theoretical curve fit to the data based on the extrapolated phase diagram of M22 solutions in an LM5 carrier. The Tm of a solution is not just a temperature but is also a measure of the concentration of the solution prior to freezing, and the latter in turn is a point on a phase diagram from which the amount of ice frozen out at lower temperatures can be predicted. The double-headed arrow indicates, at one example point, the discrepancy between the predicted amount of ice at the recorded Tm and the actual amount of ice found. In this

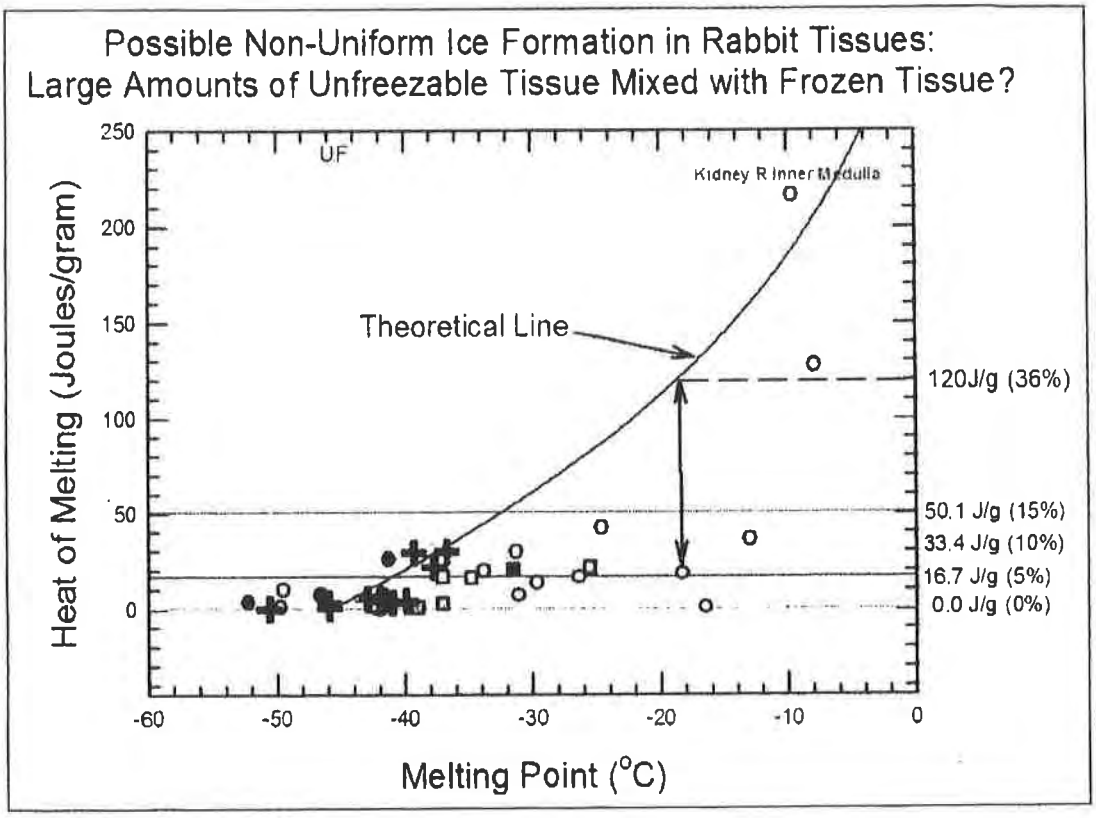

Figure 8: Estimated amount of unfreezable tissue in frozen tissue samples taken from whole rabbits prepared for vitrification with M22. Black plusses are results for pure M22 solutions with LM5 as the carrier. Blue plus signs are results for tissues collected from isolated rabbit kidneys perfused with M22 in LM5.

case, the **total amount of ice formed is a small fraction of the amount predicted**. This means that a small part of the sample must contain a much higher percentage of ice than indicated on the right and the rest of the sample must be ice-free. We are in the process of collecting more definitive data on this interesting aspect of our results as this grant letter is being submitted, and we are checking our thermogram integration method to make sure the discrepancy represented by the double arrow is not simply an error of integration. Another bit of information that follows from Figure 8 is that maximum freeze-concentration is expected to occur by about -46°C during slow freezing.

C) Kidney Vitrification

We pursued the dG method to its logical conclusion. We found we were able to move all of the impermeant polymer species into the transitional VMP-like solution employed just before cooling the kidney to -22°C without apparent problems, thereby confining the equilibration required during the actual M22 perfusion step to just the new permeant species found in M22 but not in the VMP-like transitional solution. We also found we could increase medullary equilibration further by raising the concentration at

the first concentration plateau from the original 5 molar to a new 6 molar. When this was extended to 7 molar, survival was affected negatively. **We also changed the washout protocol to accelerate the beginning of washout** because we found that the venous and urinary concentrations continued to rise for many minutes after switching from M22 to the VMP-like solution.

Much comparative analysis of solution equilibration rates indicated that a dominant factor in urinary and presumably medullary equilibration is the viscosity of the perfusate. Consequently, we replaced the PVP K12 in M22 with polyethylene glycol of relative molecular mass 1000, and, in some experiments, we reduced the concentration of X1000 by 90% or more. Both of these maneuvers had a substantial positive effect on equilibration.

We also dropped the NMF from the M22 formula and replaced it with 3-methoxy-1,2-propanediol gram for gram. This substantially reduced the toxicity of the resulting solution, which was named M222. Figure 9 shows the overall gain made in percent equilibration and renal injury using the low-X1000 version of M222 called M222LX. **Remarkably, kidneys cut in half after perfusion with M222 and M222LX were shown to attain complete medullary vitrification during passive slow cooling in our ITS device after perfusion at only 40 mmHg**, a first.

Unfortunately, the replacement of PVP, NMF, and X1000 also **weakened the glass-forming tendency of the solution substantially**, and it became apparent to us that the M222LX solution, at least, is too unstable for practical use. When half-kidneys vitrified with the new solutions were warmed back up, all experienced visually strong medullary devitrification (warming rates, ~5-15°C/min). However, when tissue samples

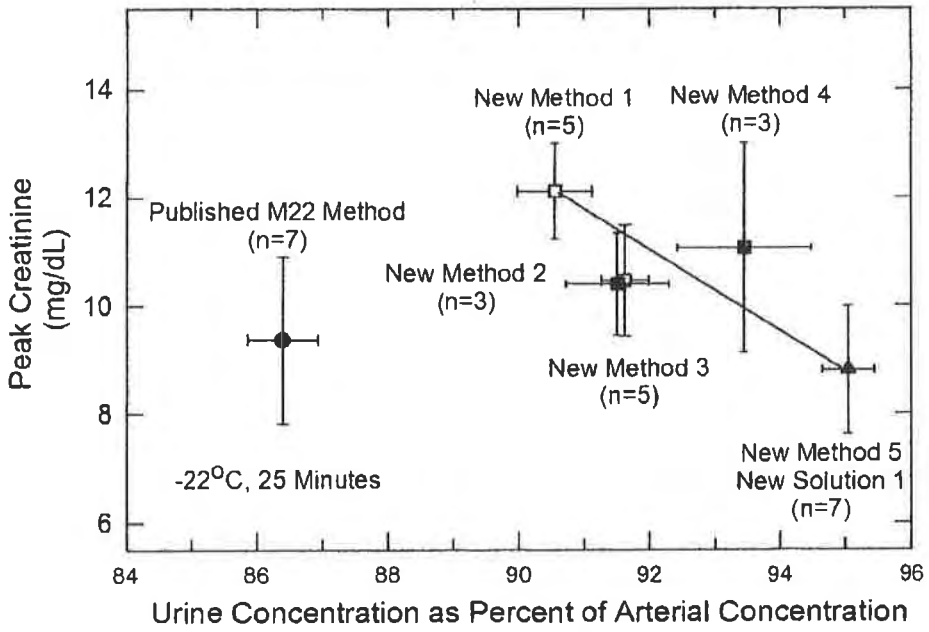

Figure 9: Improvements in kidney viability with increased percent equilibration with vitrification solution achieved to date in 2005 and 2006. "New Solution 1" is M222LX, a formula in which the X1000 level was reduced by 90% to reduce viscosity. .

from these kidneys were vitrified and rewarmed in our differential scanning calorimeter, the amount of ice formed was a strong function of warming rate, suggesting **that if we can work out a relatively simple microwave warming approach, we might be able to largely eliminate devitrification** at warming rates below 50°C/min.

Several kidneys were devoted to the task of determining, under the standard warming conditions for intact kidneys being transplanted after rewarming, **the severity and origin of injury from both medullary devitrification and surface viscoelastic stress**. These studies were carried out by cooling and warming kidneys in M222 initially using Method 5 above, transplanting them, and then photographing them 3 and 30 minutes post-transplant. These photographs included photos of the renal surface to show surface injury marked by blood trapping in the cortex and also included photos of the renal medulla in kidney cross-sections to show medullary congestion caused by vascular injury secondary to ice formation.

Cooling to -100°C did not induce either form of injury, and cooling to -110°C similarly resulted in only light injury even when the kidney was held at -110°C for the time normally required to cool from -110°C to -130°C and to rewarm back to -110°C. This indicates that **both nucleation and viscoelastic stress develop below -110°C**. Cooling kidneys to -130°C resulted in both medullary injury and surface injury, but **medullary injury was preventable** by perfusing M222 or variants of M222 containing an extra 1% GA or NMF at 80 mmHg for 35 minutes. **Our previous protocol for avoiding surface injury was no longer effective**, for reasons that elude us. Although surface injury is not fatal to the kidney, it remains a defect we would like to avoid. The fact that holding kidneys at -110°C appears to be innocuous **may provide a way for us to eliminate surface injury in further experiments**, because annealing the kidney at -110°C before completing cooling to -130°C may allow viscoelastic stress to be reduced to a non-damaging level at least during cooling.

The fact that extreme conditions of medullary loading seem to be needed to prevent medullary ice formation is sobering. Figure 9 and the visual lack of ice in kidneys after cooling made us feel our problems with medullary ice formation might be largely over before we performed the "reality check" of evaluating post-transplant vascular congestion. On the other hand, the DSC studies on vitrified kidneys mentioned above indicate that we may still be able to avoid medullary ice damage without extreme loading measures if we can accomplish safe electromagnetic warming (EMW). The fact that **major renal structural injury appears absent at -100 to -110°C** could theoretically allow us to test EMW methods for safety in non-nucleated kidneys before using them to try to eliminate devitrification.

To do this, however, we must be able to first recover kidneys from -100°C or -110°C in viable condition. This has not been previously accomplished. Further, **our attempts to reproduce our best results after cooling kidneys to -45°C have thus far been impossible to reproduce**. Kidneys can survive cooling to this temperature, but after transplantation show high creatinine peaks indicative of little safety margin of the kind we would like to have prior to cooling to -100°C. We tried to check to see if this problem is somehow due to the use of M222 vs the original M22, but when the experiment was repeated with the original M22 protocol, all kidneys failed to support life. We think this is because **we no longer have access to our original B. Braun hydroxyethyl starch**, a fact that caused us endless problems in 2004 to 2005, and the use of German or Japanese HES simply causes too much damage. However, it is also

possible that using high tonicity solutions to cool to -22°C is somehow sensitizing the kidney to further cooling, as reported in a different context by Khirabadi et al.

The answer to all of these problems may be to be true to the original principle of using **higher concentrations of weaker glass-forming agents**. We had remarkably low toxicity when we perfused kidneys with M22B in the past, whose concentration is 9.7M vs the 9.3M levels in M22, but these kidneys were much less stable against ice formation despite their higher absolute solute concentrations. If we increase the concentration of solutes in our vitrification solution still further, we may close the gap in stability and eliminate our present problems with stability while simultaneously still controlling toxicity. New possibilities for such modifications are also described below in section F), Cryoprotectant Technology.

Alternatively, we know from the studies on both kidneys and free solutions that **raising the concentration of individual ice blockers** from 0.5% to 1% greatly improves the stability of the solution against ice formation. Perhaps what we need to do is to raise specific ice blocker levels to **above 1%**. This will definitely raise viscosity and reduce medullary equilibration overall, but may still increase medullary stability on balance, without increasing toxicity.

Finally, we have also begun to study a radical approach to medullary equilibration that consists **of retrograde perfusion of the ureter** into the pelvis and from the pelvis into the medulla and then into the cortex and out the renal vein. One potential advantage of this approach is that the ice blockers would be able to gain access to the pelvis and medullary collecting duct lumens without having to first pass through the glomerulus. This may substantially increase ice blocker concentrations throughout the medulla if they are not filterable through the glomerular basement membrane. A preliminary experiment using trypan blue dye to tag the retrograde perfusate indicated that the dead volume in the medulla can be displaced within a few minutes by this technique, after which retrograde perfusion should be unnecessary unless the perfusate ultrafiltrate coming from the renal corpuscle remains deficient in ice blockers, in which case the retrograde perfusion would end when M22 perfusion to the kidney as a whole ends.

The vitrified kidney that survived in 2002 was perfused at 80 mmHg. Although we consider this pressure to be damaging, **a small advantage from 50-60 mmHg** may be a good tradeoff as well.

One beauty of the "reality check" model is that it gives results within about 30 min of transplantation. This allows candidate changes in procedure to be evaluated rapidly and with reduced animal usage. However, we must bear in mind the possibility that **what appears to be irreversible vascular stasis by this test may not always be irreversible**. In fact, it will be important to discover whether renal congestion is invariably linked to survival or is merely correlated with damage. It may not be necessary to completely eliminate surface and medullary congestion to obtain adequate functional recovery of the kidney.

Finally, although we are using survival at -50°C as a prerequisite to survival at lower temperatures, **it is not yet certain that cooling to -100°C is actually more threatening overall than cooling to -50°C**. Although there should be greater thermal stress and greater chilling injury at the lower temperature, and more problems associated with rewarming, to some extent these problems may be balanced by faster cooling rates from -22°C to below -50°C and perhaps by faster warming rates above -50°C. Therefore, if we continue to have problems getting good results at -50°C, we will explore cooling to lower temperatures anyway.

D) Cornea Vitrification

2006 has been the year in which cornea vitrification has been rigorously tested under the auspices of a Phase 1 SBIR grant from the National Eye Institute. A team consisting of Dr. Wu and Dr. Ge traveled to St. Kitts twice, once in the month of January and once in the month of February, 2006, and **transplanted several control and several vitrified human corneas into vervet monkeys.** The cornea cryo-shipper described above was used to transport the vitrified corneas without incident. All corneas were examined in vivo from time to time using a clinical specular microscope to measure corneal thickness and endothelial cell density and morphology, and the eyes were also photographed. After about 4.5 months in vivo, the corneas were retrieved and processed for light microscopy, scanning electron microscopy, and sex chromosome staining using in situ hybridization (FISH). The results showed that **the vitrified corneas outperformed the controls** in terms of visual appearance following transplantation, attaining much higher rates of visual clarity and recovering from the stresses of transplantation sooner. They also retained dramatically higher endothelial cell counts than the controls based on in vivo specular microscopy. **Therefore, from a clinical point of view, it seems clear that vitrified corneas have utility** and compete very favorably with control corneas, which cannot be exported to countries where they are needed due to damage incurred during shipping, a problem that does not appear to pertain to the vitrified corneas.

We were disappointed that our FISH assays turned out to be negative in all transplants, whether of vitrified corneas or control corneas. We had carefully validated this assay before submitting our experimental corneas to it, so we are not sure what went wrong. Experiments are in progress to determine whether the assay is affected by cryoprotectant exposure without subsequent transplantation or is affected by the dehydration we suspect may have happened during cornea retrieval in St. Kitts, or is affected by fixative temperature upon contact with the cornea and subsequent shipping, or even by vitrification and warming itself.

As of this writing, **we are still analyzing the pathological reports from our ocular pathologist partner, Dr. Nora Laver, and we are still waiting for many of her results** with SEM and histological evaluation to become available. When all of this information is processed and compiled, a progress report will be sent to the National Eye Institute and, assuming the results warrant it, a Phase 2 grant application will be prepared in which we hope to include human clinical trials as one component. Other components will include modifications of our technique to reduce corneal swelling just prior to transplantation and experiments in which corneas are vitrified in sterile containers instead of being vitrified bare in air.

We completed DSC studies on human corneas as proposed, and **verified the absence of detectable ice formation in these corneas even after slow cooling followed by slow warming (5°C/min for both).** Three of three studied corneas tested were found to have glass transitions between -110°C and -97°C.

E) Liver Slice Vitrification and Cold Storage

We continued our exploratory studies in anticipation of collaborative work with Allergan, a multi-billion-dollar company located in Irvine, California. Our results

indicated that **we could obtain in the vicinity of 100% recovery of vitrified rabbit liver slices** based on K/Na ratio and with the use of a unique technique for adding and removing cryoprotectant that would be attractive for most users. We also found that **slices stored at -135°C for 40 days were just as viable for unstored or more briefly stored slices**.

Armed with this set of accomplishments, we prepared 4 groups of preserved slices for simultaneous **testing at Allergan**, as follows. First, we vitrified some liver slices on a Monday and stored some slices in TransSend on the same day. On Tuesday we prepared fresh control liver slices in TransSend and retrieved some vitrified liver slices from our -130°C ITS storage unit after a few weeks of prior storage. All four groups of slices were then driven to Allergan for evaluation. The vitrified slices were warmed up to prove to Allison that they had been previously vitrified and stored for some period of time. **Allison checked the slices for ATP levels and weight, thickness, and visual appearance as a function of slice time in culture at 37°C from a few hours to 3 days**.

For the most part, the **slices survived and lasted for the whole three days**. The slices prepared on Tuesday and held in TransSend for only 4 hours before culturing **behaved as well as Allison's control rat liver slices**, but **ATP levels were depressed after vitrification and after 24-hour cold storage** in TransSend in comparison to the fresh (Tuesday AM) slices. We were somewhat disappointed because our in-house assays from the same experiment indicated full recovery of K/Na ratio in all treatment groups, but Allison was thrilled that the slices survived at all.

Based on this experience, we **developed an in-house HPLC assay for ATP** at 21CM. Although our results are based on just one kidney slice experiment and one liver slice experiment, it appears that a recent modified method for adding and removing M222 yields **about equal percentage recovery of K/Na and ATP** after vitrification and recovery of adult rabbit liver slices, which means that if our K/Na ratios are high, Allergan's ATP levels should be high as well, which fails to explain the discrepancy between our original in-house results and the ATP deficiencies seen at Allergan. We think the difference may be that **Allergan uses a minimally-supportive culture system whereas we use a maximally-supportive tissue bath system, and our slices are probably too thick for Allergan's culture method.** However, it's also possible that some of the discrepancy could be caused by random variations in experimental outcome, something we hope will be reduced as we obtain more experience.

Dr. Vickers has recently expressed her desire to do **more joint experiments**, and we propose to proceed with these studies with Allergan for the balance of 2006 as time is available. These studies would involve replication of our recent work with the modified vitrification procedure using M222 and, as may be called for, variants that may include such solutions as MEG, E22, M22B, VeGD, and other possibly **less toxic vitrification solutions**. On the other hand, we may use mostly **modified cryoprotectant introduction and washout methods** based on **a new theoretical model** developed by Dr. Fahy for studies on ova and stem cells (see below). In the longer run, **we may need to acquire a precision tissue slicer** in order to make slices thin enough to support properly in a culture model, as may be the norm for pharmaceutical studies.

The importance of these experiments is considerable because they in principle offer us access to large markets with a short time horizon and without FDA hurdles to overcome. As one notable example, recent discussions between Mr. Barry and Eli Lily have indicated **an immediate interest on the part of Eli Lily** in buying vitrified slices from us once we have demonstrated their functionality and integrity.

F) Cryoprotectant Technology

As noted above, subtracting 0.9% w/v X-1000 from M222 significantly reduced the stability of the solution. In 2006 we found that **replacing the missing X-1000 with 0.9% lactamide** or even adding an additional 1.2% lactamide (total lactamide, 2.1% w/v) to raise total solution concentration to 66% w/v yielded **a solution equal in toxicity to M222LX but with greater stability against ice formation than M222LX.** Replacing half of the 3-methoxy-1,2-propanediol (MP) with lactamide, with or without the addition of an extra 2.1% lactamide, slightly reduced viability in our kidney slice model based on the K/Na ratio assay. These experiments raise useful possibilities for modification of our kidney perfusate to achieve greater stability without increased toxicity.

Ice stability testing in vitro revealed that when 0.9% N-methylformamide (NMF) was added to M22 diluted to 90% of full strength (90% M22), **it was more effective in restoring solution stability against ice formation than adding 0.9% X-1000**. This indicates that **the subtraction of NMF from M22 is a major factor in the reduced stability of M222 as compared to M22.** Retaining some NMF, adding some agent other than 3-methoxy-1,2-propanediol (MP) in its place, or even increasing the amount of NMF may be more strategic than replacing NMF with MP as in M222, once the right balance has been found.

Several alternatives to NMF were tested. The rank order of effectiveness in preventing ice formation in 90% M22 was: NMF>GA>NMA>urea>0.2X LM5>lactamide. It is surprising that GA and urea were as effective as they were in this experiment, and GA would be a particularly attractive candidate in having remarkably low intrinsic toxicity and viscosity that is probably lower than that of MP.

We continued **amide toxicity neutralization experiments**, now moving on to NMF and dimethylformamide (DMF). 20% w/v solutions of both amides were non-toxic, and 20% NMF plus 20% DMSO had essentially the same toxicity as 20% formamide plus 20% DMSO, but, not unexpectedly, 20% DMF + 20% DMSO was very toxic. This experiment raises **the intriguing possibility of substituting the much more stable NMF in place of the customary formamide in M22 or M222** to create a much more stable but no more toxic solution. In combination with replacement of some MP with GA or NMA or urea to further reduce viscosity and increase stability, additional gains may be possible. This titration experiment did not reveal a concentration of NMF that was toxic by itself, nor did it establish whether the addition of DMSO to NMF reduces NMF toxicity as is the case with formamide and urea. Completion of this titration curve will be necessary in follow-up experiments.

The reason we developed a novel cryoprotectant (GA) for use by Genzyme Biosurgery was due to Genzyme's concern over objections to the use of formamide. To gain further support for our agent's lack of toxicity, **we plan to infuse a GA solution intravenously into rabbits at normal body temperature** to verify the lack of any obvious untoward effects.

G) Vitrification Physics

Ice Control

Expansion of Commercial-Scale X-1000 Production. X-1000 is the polyvinyl-alcohol-based ice blocker that is commercially sold by 21st Century Medicine. Is it sold as a 20% solution, and produced in-house. The advent of the use of 21CM vitrification solutions in 2005 for whole body human cryopreservation by the Alcor Life Extension Foundation has caused orders of unprecedented size to be placed for X-1000. Additionally it was decided to begin using commercial-grade (non-aldehyde-terminated) X-1000 for all further in-house research. Previous production procedures that resulted in a 6 liter lot size were insufficient to meet this demand. A new production procedure resulting in 24 liter lots was therefore devised, validated, and documented to enable 21CM technical staff to produce this product in sufficient quantities and on a less frequent basis.

Molecular Mechanisms of PVA Inhibition of Ice Development. A paper on molecular mechanisms of inhibition of ice formation by polyvinyl alcohol (PVA) was completed for the journal cryobiology. Early versions of this paper were rejected by the journals Nature, Science, PNAS, and JACS back in 1999 and 2000. The work was instead submitted as an abstract and presented orally at the 2000 meeting of the Society for Cryobiology meeting in Boston. Part of the work was published in the paper, "Inhibition of bacterial ice nucleation by polyglycerol polymers," in the journal Cryobiology in 2002. However, the molecular modeling portion of the work has been overdue for publication as a full paper.

A literature search was conducted to bring the references up-to-date, and it was discovered that other researchers have in recent years published proof that PVA interacts directly with ice. In particular, the polymer has been found to cause both thermal hysteresis and powerful recrystallization inhibition. 21CM holds a patent claim on the use of PVA for recrystalization inhibition, the claim having been made years ago based on PVA's efficacy as a general ice growth inhibitor, and likely direct interaction with ice.

The paper to be published in Cryobiology will emphasize that PVA is a stable and cheap synthetic analog of anti-freeze proteins, capable of performing to various degrees all functions previously thought unique to anti-freeze proteins.

Fracture Avoidance and ITS Elaborations

Defining Conditions for the Avoidance of Fracturing in Rabbit Kidneys. We previously reported that we had discovered a procedure by which rabbit kidneys could be immersed in liquid nitrogen and recovered without any fracturing. To define the limits of the conditions for fracture avoidance, we needed to also establish conditions that do cause fracturing. In 2006 it was discovered that if a kidney near the glass transition temperature (-124°C) is cooled to -150°C relatively quickly (in 30 minutes), and then suspended in -175°C nitrogen vapor relatively briefly (40 minutes), and then rewarmed for one hour at -145°C and then for 15 minutes at -135°C before transfer to 0°C vapor above a pan of water ice, the interior of the kidney is found to be fractured. Therefore, **recovering the rabbit kidney from liquid nitrogen temperatures without fracture injury is only possible with careful multi-hour cooling and rewarming protocols** to allow relief of thermal stresses during cooling and rewarming.

Toward a Commercial CIVS Unit Production Capacity. To avoid fracturing of large organs, or small organs when complex cooling and rewarming protocols are not practical, storage at temperatures warmer than liquid nitrogen is required. We have named this type of storage Intermediate Temperature Storage, or ITS, and 21CM

commercial ITS devices are known as CIVS (Controlled Isothermal Vapor Storage) units. A CIVS prototype constructed from an MVE-1411-equivalent dewar is currently in use at 21CM for storing tissue at a constant temperature of -135°C.

As noted above, a larger CIVS unit based on the MVE-1841 dewar was designed and proposed to the Alcor Life Extension Foundation in 2005 as a storage unit suitable for storage of human "neuropatients." In 2006, Alcor contracted with 21CM for the construction of this unit, and **21CM successfully subcontracted the work out to The Aubrey Group**, a biomedical engineering firm in Irvine, CA, to construct the actual unit with the hope of developing a relationship and infrastructure for larger scale production of CIVS units on a commercial basis.

Ice Control

We improved our new **workhorse supercooling system** for discovering new ice blockers by eliminating bare metal thermocouple probes and replacing them with plastic-encased probes. After running many candidates, we still have not bested dG as a high temperature anti-nucleator. We will continue these experiments whenever our technologists have spare time to pursue them until new and effective high-temperature, high volume (~46 ml) antinucleators are discovered. David Ta, who was doing these experiments, is succeeded by Lenetta Griffin as the main experimentalist for this work.

H) Kidney Cold Storage (TransSend)

Answering Reviewer Questions about TransSend Efficacy. As noted above, our Phase II SBIR grant application was turned down by NIH because one of the reviewers was confused about whether TransSend is really superior to UW solution and about whether it is necessary for TransSend to be superior to UW solution in order for it to be commercially and clinically valuable and successful. Although we explained that equivalence to UW solution efficacy is sufficient, greater clarity was needed about the superiority of TransSend to UW solution in the rabbit model. We therefore conducted several transplants that allowed us to, in part, better compare TransSend to UW solution. These experiments showed **consistent superiority of TransSend**, and caused us to discover that the reason for a huge baseline shift in our cold storage results between 2002 and 2003 was the institution of postoperative hydration in the 2003 experiments, which had a large and reproducible detrimental effect on renal recovery, contrary to earlier published experience in the United Kingdom. We also discovered that the total flush volume used prior to cold storage is critical, 100 ml given at 60 mmHg being necessary to adequately protect the rabbit kidney in comparison to only 60 ml.

Seeking Cytoprotection from Erythropoietin. Based on evidence in rats that erythropoietin has profound cytoprotective effects against warm ischemic injury by raising heat shock protein levels, we tested the use of erythropoietin (Procrit) incorporated into TransSend and the use of erythropoietin (EPO) in vivo given about 24 hours before nephrectomy in separate experiments. When EPO was used in TransSend, the results were superb in one transplant, normal in another, and poorer-than-normal in the third. When EPO was given in vivo, again **no consistent beneficial effect was seen**, and detrimental effects may have been present. It remains possible that lower doses of EPO, whether in TransSend or in vivo, would avoid negative effects and thereby unmask positive benefits.

Testing TransSend in a Canine Total Body Washout and Profound Hypothermia Model. TransSend is a more advanced formula than the MHP-2 solution that has often been used by cryonicists for transporting patients to Alcor for cryoprotectant perfusion. It therefore seemed timely to test it as a possible replacement for MHP-2. In addition, it was important to 21CM to establish that TransSend is not damaging to other organs in the body, so that its adoption as a whole-body organ preservation solution for clinical transplantation can be facilitated. Consequently, 21CM partnered with Critical Care Research, Inc. in order to perform a total body washout and profound hypothermia experiment with TransSend and to compare the results to MHP-2. We found that the only solution that had actually been tested in this model in the past was MHP-1, a simplified version of MHP-2, so we used this solution to establish an initial baseline. **Both dogs recovered from 4 hours of continuous perfusion at about 7°C, and we found less fluid uptake into the dog during this perfusion when TransSend was used in place of MHP-1. Cerebral recovery was also more rapid postoperatively with TransSend. Therefore, it appears that TransSend is at least not more hazardous to use than MHP-1,** but because improvements in technique were instituted in the TransSend experiment, and because a 4-hour perfusion time may not be adequate for isolating differences between most cold storage solutions, we can't yet say that TransSend is superior to MHP-1 or MHP-2. However, we can say that **TransSend is able to preserve the heart for a period equal to or longer than the current clinical limit, and that it also preserves all other sensitive organs such as the lungs and the pancreas very well for times that have clinical relevance for transplantation.** Therefore, this experiment is a good first step toward showing the suitability of TransSend for multiple organ procurement, and we hope to repeat and extend this experiment in follow-up studies. This experiment addresses also several issues pertinent to re-submission of our Phase II SBIR grant for the development of TransSend as a viable clinical preservation solution.

Extending TransSend Components to a Warm Ischemia Protection Model. Some time ago, we suggested the use of intravenous polyethylene glycol prior to the institution of warm ischemia in rabbits as a way to preserve their perfusability after subsequent cold storage. This idea was taken up by Critical Care Research and forms the basis of their "hot shot" formula. We felt decaglycerol might be effective also, and mentioned this in our original TransSend patent application. Because of this mention, we were able to file a second application to cover this use, and we carried out experiments in which dG was given before cardiac arrest lasting one hour, after which the animals were perfused with fixative for ultrastructural follow-up. To complete the picture for a product with clinical efficacy, we also **infused 8 ml of dG solution intravenously into rabbits and allowed them to recover as a toxicity control. No untoward effects were observed.**

I) Cardiac Cold Storage and Intermittent Perfusion

Blood Perfusion. We completed the software and hardware improvements needed to make our human heart perfusion machine functional. We initially tested our system using several **monkey hearts** obtained from St. Kitts in conjunction with the collection of corneas from the same animals under our NIH-funded cornea vitrification grant. The first four monkey hearts arrived frozen and therefore non-functional, but served as useful models nonetheless. Three more monkey hearts were received from St.

Kitts on three successive days and were perfused with blood with the help of Tingchung Wang from the University of Rochester. We experienced technical problems with all of these hearts, but found that **monkey hearts preserved for 24 hours with UW solution were alive and able to beat and respond to inotropes at 37°C** in our blood perfusion circuit **and to attain physiologically normal levels of cardiac output**. One of our biggest problems has been bleeding due to damage in the vicinity of the atrioventricular junction.

We were able to obtain **our first human heart preserved with URS at the University of Rochester and stored for over 24 hours** before blood perfusion. We did not realize at the time that this heart never returned to 37°C and did not beat consistently for that reason. We were able to get it to beat in response to inotropes, and it was definitely soft and not in rigor, which is what happens when hearts exceed their period of viable storage normally, but we continued to have bleeding problems and were not satisfied with the sustainability of the beating. However, when we immersed the heart in warm fixative, it began to beat again! We then realized that the heater in our circuit had not returned the heart temperature to 37°C and that it is essential to allow the heart to reach normothermia for sufficient time to recover before it is concluded that the heart cannot resume adequate function.

Finally, we were able to obtain one fresh **dog heart** from Critical Care Research and perfuse it with blood in our system. We learned much about the mechanics of heart perfusion from this experiment and were able to observe **tremendous cardiac functionality after successful defibrillation**, but we continued to have problems with bleeding, and it was clear we needed to make additional changes in our perfusion equipment. These fairly extensive changes have now been completed, and we expect to obtain and perfuse additional human hearts from standard commercial sources before the end of 2006 as required by our grant.

Heart Transplantation at Critical Care Research. We decided **we can not rely on UR to do its part of our grant-supported research successfully, and that it would be up to us to try to do dog heart transplants locally**. For this work, we obtained the cooperation of **human heart transplant surgeons at Loma Linda**, who have volunteered to come to Critical Care Research and help us by doing these transplants pro bono. We are very excited, and our first attempted heart transplant was nearly successful. The whole body hypothermia experiments referred to above allowed us to refine our skills with cardiopulmonary bypass in anticipation of our next transplant attempt, which will take place before the end of the year. All members of the team from both Critical Care Research (CCR) and 21CM are energized about these experiments and believe we can beat UR's performance. We plan to invite the key UR personnel to visit us and witness our methodology when we are ready to demonstrate success to them. However, **our intention is to petition NIH to move the venue of the transplant experiments from UR to CCR**. We are extremely impressed with the skills and personality of our main Loma Linda surgeon, Dr. Nahidh W. Hasaniya.

Total Body Washout and Profound Hypothermia with URS. On Friday, October 13th, 2006, CCR and 21CM performed total body washout and profound hypothermia with URS, maintaining the protocol the same as prevously used for TransSend except for **dramatic improvements in systemic pressure enabled by the use of negative reservoir pressures to improve venous return**. The dog was eating solid food by October 17th and generally seemed to recover well except for the possibility of some allergic reaction involving puffiness around the eyes and elsewhere.

This experiment has value to us in **emphasizing the compatibility of URS with multiple organ donation, verifying the ability of URS to preserve the canine heart for a clinical period of time in vivo, and helping us interpret the physiological requirements for supporting whole animal viability during profound hypothermia.** The results are compatible with Haneda's use of blood to preserve dogs for 3 hours at similar temperatures and with the clinical observation that simple crystalloids such as Ringer's solution can preserve human kidneys for 6 hours despite the very unfavorable composition of such solutions. As noted above, the current protocol may need to be extended to longer periods of cold perfusion to allow differences in solution efficacy to become apparent. **We are already preserving canines longer than any other laboratory ever has, and will break all records if we can extend the model to 8 hours of preservation or longer.**

J) Hepatocyte Cold Storage

We did no further work on isolated hepatocytes in 2006 and do not plan further experiments on these cells unless approached by an interested party.

K) Contract Research

We will not be doing paid contract research for the balance of 2006 of the kind accomplished with Genzyme in the past. However, we do plan to be engaged in research under the auspices of enabling contracts with Cornell University, the University of Arizona, and Tecnobios Procreazione. Although we will not be paid for this research, this research may lead to **clinical deployment of our technologies in large international markets with extreme speed.**

The most significant of these contracts in terms of both research scope and clinical scope will be the work done with Cornell University under the auspices of Roger Gosden. This work will apply 21CM formulas and, perhaps, 21CM methods for the preservation of human ova, human embros, and human stem cells in **a high-impact, high-visibility medical center. Embryos vitrified with 21CM formulas may very well be used to make human babies.** Ova cryopreserved with 21CM formulas will be evaluated for competency to make babies and, if found promising, may be used clinically. It is unclear what will be done with human umbilical cord stem cells, but any results obtained are likely to be useful for the commercial application of our formulas for this market.

A major issue for the proposed studies will be methodology. Dr. Fahy has invented potentially very valuable methodology for vitrification that would be appropriate to apply to the Cornell projects, but John McGrath, an MIT-educated engineer from the University of Arizona, will be asked to design the methods for this project to **avoid potential conflicts of interest with work to be done in Italy** (see below). This may be inappropriate for us, and **we may decide to use 21CM methodology for the Cornell studies** in order to establish our methods in this major center and not just in Italy. Our contract with the Italians does give us the rights to use our methods outside of Italy, so **in principle no conflict of interest should exist.**

The prospect of 21CM formulas and perhaps methods being used to make human babies raises some considerations of **liability in the event of birth defects.** While this risk is minimal or non-existent based on all preclinical and clinical research to date over

the last 3-4 decades, including research done with VS1, a formamide-containing solution used to vitrify mouse embryos in 1985, **we are likely to favor the solution developed for our research with Genzyme, VeGD, which lacks formamide**, for the initial experiments at Cornell. Formamide is known to interact with DNA, although it is not mutagenic and is not carcinogenic, whereas there is no such information about GA, the unique ingredient of VeGD. GA is closely related to derivatives of GA that are widely used for the formulation of pharmaceutical agents that are ingested routinely, and thus **should have presumed non-toxicity for cells even at normal body temperatures**. We will verify this as mentioned above by infusing GA into rabbits, and may seek collaborative partners to test GA for animal embryo vitrification as well, to further verify the safety of our formula(s) for embryos and/or ova specifically. We should also be protected by the Cornell imprimatur, Cornell sharing responsibility with us for the decision as to whether cells treated with our solutions are safe to give to patients.

The other major studies will be those done in Italy using solutions and methods designed entirely by Dr. Fahy at 21CM. These studies will be limited to the vitrification of human ova. Initial studies will simply verify that ova look morphologically normal after treatment with our solutions and after vitrification and rewarming, and that there is no obvious reason not to use them to make babies. **Once we get to this stage, the ova will be fertilized and used to make babies**.

Dr. Fahy has already documented the basic method he plans to use in these studies in the form of an attachment to an email sent to Mr. Barry and Mr. Horswell before his first phone call with Dr. Gosden after the signing of our contract with Cornell. Undoubtedly the finer points of the method will evolve based on experience, but the basic principles are hoped to be sound. **The methodology will allow sterile packaging of vitrified ova (and other cells) and reproducible procedures that do not require highly skilled technologists or great manual dexterity to perform**. The goal will be to attain a much higher ratio of live babies to vitrified eggs and much more reproducible results.

It is uncertain how much of this work will be performed in 2006. Most work not done in 2006 will be completed in 2007.

These contracts are the most significant ones entered into by 21CM so far in terms of the potential immediate impact on company sales and visibility.

L) Collaborative Cryopreservation Research

We are also very excited about our continued collaboration with **Allergan** and our expected collaboration with Advanced Cell Technology and CHORI through the possibility of a grant from the **California Institute of Regenerative Medicine (CIRM)**. Our potential work with Allergan has been described above.

Our work with CHORI and ACT is expected to involve **the use of 21CM solutions as freezing solutions for ACT's unique cells**, which are better poised for therapeutic use than most stem cell lines in the pipeline. **The grant will use Dr. Fahy's approach to the rational design of freezing procedures**, an approach he outlined at the 2nd International Conference on Cryopreservation of the Human Oocyte in October, 2006, and in a symposium publication expected to result from that meeting. Essentially, the osmotic limits of ACT's cells will be measured, and phase diagram information will be used to **calculate the concentration of cryoprotectant required** to keep the cells from exceeding the hypertonic limit during freezing. Methods for adding and removing

cryoprotectants will also be designed based on the known osmotic limits and on measurements of the permeability of the cells to three candidate cryoprotectant solutions, the methods being designed to keep cell volume within the cells' osmotic limits. The **optimal cooling rate will also be calculated** from measurements of the cells' permeability to water. The results of this approach will be compared to those of using the Cryo-Stor formlas of BioLife Solutions, a competing product line now being sold through VWR that consists of 5% DMSO plus apoptosis blockers. Our involvement in any follow-up studies, including studies involving vitrification, will be dependent on how these Phase 1 studies come out, and on how well 21CM methods do in comparison to BioLife's methods.

Research on the CIRM project will not begin until funding is received in 2007, should the grant be funded. All of the research for this project will be done at CHORI and at ACT, with possible visits by Dr. Fahy to troubleshoot and jump-start the experiments. **We hope to keep the time commitment by 21CM personnel minimal.** By the time the project moves on to vitrification, should it ever do so, 21CM should already have patent protection pending on its VeGD method and other related methods. It may also have patents for DMSO-free cryopreservation solutions that may be of particular relevance for the cryopreservation of stem-like cells, which may be easily and undesirably differentiated into unwanted cell types by DMSO.

University of Notre Dame. We expect to continue low-level collaborative efforts with Dr. John Duman at UND. Most or all of the needed results have already been obtained, and we will work with Dr. Duman to ensure that they are published with adequate and accurate documentation. Dr. Duman's studies with us indicate that something akin to vitrification is the method by which some of the most hardy Alaskan insects pass the winter unscathed, and therefore support our approach to cryopreservation by vitrification.

GUIDE. We have agreed to consult with Inge de Graaf on a project to vitrify organ slices in the Netherlands. These slices will be evaluated using gene expression profiling and other end points and will probably result in fascinating new insights into the biological effects of our specific cryoprotective cocktails as well as of cooling and warming in the absence of ice formation. 21CM has been promised the right of first refusal to any commercial spinoffs of this work. The expression profiling will be done by a giant biotechnology company, Organon, which will become familiar with 21CM technology as a result of this study, which we consider a significant development.

Other. We recently re-established contact with **HepaHope**, which expressed eagerness to begin working with us. We are less excited about working with them based on their past business record, but are open to potential work if they can pay for it and can convince us it is worthwhile. We met several parties at the **World Transplant Congress** in Boston who expressed varying degrees of interest in working with us, but we have no immediate plans to work with any of them although we will keep an open mind about this.

M) New Initiatives

Our experience with kidney vitrification and whole body vitrification to date clearly indicates that **the kidney is perhaps the most difficult organ or tissue in the whole body to vitrify** with the possible exception of the eye. We have developed technology so powerful that it should be far more than equal to the task of vitrifying

whole organs, and yet we continue to require additional work on the kidney in view of the unique structure of this organ and the problems this poses for medullary vitrification.

This situation has made us realize that **there may be much more immediate opportunities for success in the vitrification of other organs**. We are in the process of preparing an analysis of how to prioritize organ cryopreservation targets based on likely ease of cryopreservation, likely market need, and likely difficulty of transplantation or experimental evaluation. However, at this stage, two attractive targets suggest themselves.

The first of these is the intestine. This is the only organ so far reported in the literature to survive **freezing and storage in liquid nitrogen** followed by vascular transplantation and subsequent life support in a large animal model. This feat was **accomplished in 1974 using low concentrations of DMSO** as the cryoprotectant and can undoubtedly be surpassed using more modern approaches. The clinical need for intestinal transplants has been revolutionized recently by advances in immunosuppression and surgery, but the intestine is highly immunogenic, which implies the value of improved tissue matching or tolerance induction based on the ability to bank the intestine. Intestinal transplants can make a large difference in the quality of life for many human children, and therefore would have positive publicity implications for 21CM in addition to the usual advantages of successful adoption by the transplantation community. In addition, it is possible to live without the intestine, so failures would not need to result in the death of recipients, a big plus in being able to establish organ cryopreservation as a clinical modality. Finally, vitrification is probably not necessary for success, which means simpler methodology and much greater margins for error in clinical deployment. Success is almost guaranteed, and should come rapidly, leaving marketing as a more limiting factor than research, which for us would be a highly unique and highly desirable situation. In addition, the likelihood of success, the clear need, and the existence of a definable market should make grant support for intestinal cryopreservation feasible.

Dr. Jun Wu, our present transplant surgeon, has expertise in this area and is writing a protocol for transplantation of rabbit small bowel segments. We hope to have this protocol approved and tested before the end of 2006.

We are not the only ones interested in the clinical deployment of frozen organs. **Dr. Amir Arav, who has recently published a number of papers on the freezing and transplantation of ovaries using his patented directional freezing method, is now in human clinical trials at Yale University for the transplantation of frozen human ovaries**. Like the intestine, the ovary is not necessary for life, and therefore failures do not have the same significance as the failure of a heart or liver or kidney. Partly to avoid competition with Dr. Arav, we do not propose to freeze or vitrify ovaries at the present time, but if Dr. Avav is successful, his success may make it look like 21CM has fallen behind unless we have similar successes in development.

The second alternative organ we have in mind is **the heart**. It will require vitrification and will be much more difficult than the intestine, but should be much easier than the kidney, and our intensive efforts to build blood perfusion machine assay methods for hearts can be used to evaluate vitrified hearts without the need for transplantation. We will not be able to consider such research in 2006, but **it remains a longer term goal** to be examined as we develop the time and the resources to pursue it.

9. Summary of Near-term Commercial Opportunities

21st Century Medicine remains committed to the ultimate goal of long-term preservation of human cells, tissues and organs, eventually bringing fundamental changes to the field of organ preservation and ultimately enabling suspended animation. To support these goals while increasing its' research capacities, the company is focused on bringing related technologies to market that are positioned for more near-term commercialization. It has become evident that the markets for our current medical offerings are too small monetarily to support a concerted manufacturing effort without the addition of a technology closer to rollout that applies directly to a larger market. Our surveys have identified the drug discovery and development market as an initial target with a market size in excess of 20 billion dollars in totality, and as a product penetrable market of almost 3 billion dollars. During mid 2005 and continuing through 2006 many contacts have been made with commercial research institutions, biopharmaceutical houses, and government agencies regarding 21CM's growing expertise in the preservation of cells, tissues and organ slices for pharmaceutical research use. Following is a list of those presentations.

NASA Office of Biological and Physical Research
OBPR RESEARCH CONFERENCE
Cell and tissue preservation capability presentation
April 4, 2005
St Louis, MO.

NAICS Physical Engineering and Life Sciences Conference
Cell and tissue preservation capability presentation
April 9,2005
St Louis, MO.

Admet, Inc.
Cell and tissue preservation capability presentation
August 14, 2005
Duram North Carolina

DARPA Technology Transfer Symposium
Dr. Joseph Prancrazio, Chair
Neural tissue preservation capability presentation
August 16, 2005
Bethesda Maryland

American Association of Pharmaceutical Scientists
(AAPS) Annual Meeting and Exposition
Organ slice business model presentation
November 2, 2005
San Antonio, Texas

NIH-CAP presentation by teleconference
Organ slice business model presentation
January 24,2006

Pfizer initial teleconference presentation
Organ slice business model presentation
February 16, 2006

Eli Lilly initial teleconference presentation
Organ slice business model presentation
February 28,2006

Merck Pharmaceuticals initial teleconference presentation
Organ slice business model presentation
March 6, 2006

LARTA Biomedical Business Venture Forum I
Organ slice business model presentation
March 14, 2006
Dallas, Texas

LARTA Biomedical Business Venture Forum II
Organ slice business model presentation
April 6, 2006
Los Angeles, CA.

Intervet, Inc.
Tissue slice cryopreservation capability presentation
May 26, 2006
Houston, TX.

Southern California Biomedical Networking Forum
Organ slice business model presentation
June 7, 2006
Irvine, CA

NIH Life Sciences Showcase
Organ slice business model presentation
June 29, 2006
San Jose, CA.

Bayer Phase 4 grant proposal presentation
In support of canine heart transplant model at CCR
July 12,2006
Dallas, TX.

Eli Lilly Biomedical Research Institute (Lilly Accelerator)
Tissue slice cryopreservation capability presentation

August 18, 2006 and August 21, 2006
Albany New York, and Raritan, New Jersey

Charles River Laboratories / Kendle International
initial teleconference - organ slice business model presentation
September 25, 2006

Partly because of these presentations, and exposure, 21CM is now being increasingly contacted by major research and commercial entities with considerable resources and interests in major commercial markets. Some of these leads could produce revenue for the company in a surprisingly short time.

Whereas in the past we sought out commercial partners with limited success, we now find that potential partners are initiating contacts with us more frequently than we are seeking contacts with others, an encouraging trend. 21st Century Medicine is quickly reaching an important threshold in terms of near-term product development. The next step, demonstration of practical application for defined products under customer defined conditions, should be followed immediately with sales to industry users.

We are very pleased to have the attention of multi-billion-dollar corporations such as Allergan, Eli Lilly, and Cook, the latter two of which could begin purchasing product almost as soon as we demonstrate efficacy in the vitrification of liver slices and ova, respectively. We are also encouraged by our very early success with a traditionally intractable system, the liver slice, and hopeful that significant demonstrations of utility may be feasible if not in 2006, then in 2007. Having Allergan as an ally and advocate is very fortunate for us, and we plan to work with them closely to demonstrate the utility of our vitrification technology for the pharmaceutical industry and general research market. There seem to be no truly competitive products to our liver slice product, so again the key requirement is proven efficacy, and there are no FDA barriers to sales.

We are excited about the **prospects for rapid development of interest in 21CM products and methods in the area of reproductive medicine** as a result of our planned research with Dr. Roger Gosden at Cornell, Dr. John McGrath at the University of Arizona, and Dr. Andrea Borini of Tecnobios Procreazione. The number of women with an immediate need for ova cryopreservation may be close to 1 million, which would imply considerable revenue to 21CM if we were to realize a $1000 royalty per woman, which is a small part of the costs of attempting to have a child from a cryopreserved ovum today. The market for cryopreserved human embryos is well established, and the possibility of significant market penetration exists for a technique that is much simpler than current methods but has similar reliability. At present there appear to be few if any regulatory hurdles between us and these markets, and if we can demonstrate success, it is likely to happen by proving clinical efficacy directly, a heady prospect.

It would be not only of high prestige to 21CM to obtain a grant from CIRM, but also of **considerable potential commercial value to be chosen by ACT as the provider of cryopreservation technology for its proprietary cells**. These cells seem to very advanced compared to the pending stem cell technologies of others, and ACT is applying for broad patent protection that could be very valuable. In addition, it would be a pleasure to support a technology with potentially broad power to ameliorate age-related changes in humans. We are bravely pitting our technology against the best conventional cryobiology can offer under circumstances that will not necessarily favor us, but if we

succeed under these conditions, we may not need to worry about competition from other approaches for a good while.

We are now in the strange position of having developed a successful process for the vitrification of cartilage, which has potentially large commercial markets, but having no clinical partner to carry this advance for us into sales. Dr. Amir Arav, who is in clinical trials for the transplantation of frozen human ovaries, also claims his directional solidification process works for the freezing of cartilage and that his process is now being used clinically. **His example may help us find a way of moving forward** with further clinical testing and selling of our already established technology without Genzyme Biosurgical.

Other relatively near-term opportunities exist for the deployment of **vitrified brain slices and vitrified human corneas.** We presently lack development partners for both, but have reason to believe that we will find allies in the Department of Defense as well as companies like Merck for the former, and have had expressions of interest by Tissue Banks International for the latter. Additional refinements in both of these potential product lines are currently being made with financing provided by the National Institutes of Health, and we are excited about the prospects for commercializing both systems in the next few years.

TransSend and URS continue to move forward, but will require additional time to establish and get through the FDA approval process.

In summary, our technologies are being given serious attention by a variety of viable development partners, and are succeeding across the board. The prospects for commercialization of 21CM technologies have never been brighter, and we intend to pursue all of them vigorously.

Summary of Terminated Initiatives

T-cubed, a distributor of human hepatocytes for research uses that tested our TransSend formulations for these cells with positive results, was purchased by another company with different commercial interests.

The possibility of vitrifying delicate cells for PerkinElmer that arose in 2005 did not materialize, for reasons that are not clear to us.

The communications we exchanged with NASA in 2005 did not lead to new business for 21CM in 2005 or 2006.

As noted above, commercial opportunities with Agrigenesis failed to materialize due to the inability of their protein to compete effectively with or add value to X1000.

We heard no more from John Morris in the UK about an opportunity for commercialization of supercooling in the food industry, and since our attempts to develop new ice blockers were not successful, there was no further basis for a relationship with him.

All efforts to elicit responses from thoracic surgeons at Hoag Hospital in Newport Beach about the possibility of using TransSend for the short term preservation of blood vessels (saphenous veins) removed from patients' legs for use in coronary artery bypass graft surgery failed.

No deals were reached with the Duke University Medical Center to cryopreserve the bioartificial blood vessels they have been developing.

We elected not to pursue further work with VetStem.

10. Non-LEF Funding/Revenue

Federal Research Grant Funding

A Phase 2 SBIR award (2 R44 HL066813-02), "**Extended Cardiac Preservation,**" funded at $835,000 for cardiac preservation by simple cold storage using University of Rochester solution. This grant was funded by the NIH National Heart, Lung, and Blood Institute. The period of this grant was from July 1, 2004 through June 30, 2006, however a no cost extension has been granted to extend the period for an additional 12 months.

A Phase 1 SBIR award (1 R43 NS053111-01), "**Neural Tissue Vitrification,**" was funded at $184,915. This grant was funded by the NIH National Institute of Neurological Disorders and Stroke. The period of this grant is from May 01, 2006 through April 30, 2007.

A Phase 1 SBIR award (1 R43 EY015977-01), "**Cornea Preservation by Vitrification,**" was funded at $195,024. This grant was funded by the NIH National Eye Institute. The period of this grant is from September 30, 2004 through Sept 29, 2005, however a no cost extension has been granted to extend the period for an additional 12 months.

Additional NIH grant submissions are planned for 2006. The earliest funding dates of these applications would be in the second six months of 2007.

Commercial Research and Product Revenue

21st Century Medicine continues to sell preservation formulations to private corporations and universities for research purposes. While the volume of these sales is small, interest in this product line is growing. Additionally, final engineering for the CIVS storage/transport systems is scheduled for completion by the end of 2006, and these systems may move into initial market introduction in 2007. Commercial contract research, such as the Genzyme cartilage preservation project, is limited but may expand as the efficacy for preservation of specific tissues is demonstrated.

21st Century Medicine also receives revenue from licensing of its preservation technologies to cryogenics organizations.

11. Comments Concerning the 2006 Operating Budget

The attached budget is organized in sections. These sections detail planned expenditures by defined account within each general budget category. The budget does not include information concerning NIH grants or commercially sponsored research.

Consideration of recent funding of the Whole Body Preservation project is also not included. In August 2006, $100,000 was received for this purpose and is currently being spent on expansion of laboratory facilities, with additional amounts planned for employee

recruitment and equipment. Further detail concerning this project will be submitted within the 2007 research proposal.

Personnel costs continue to rise modestly, although a change in benefit administration suppliers has helped reduce employee insurance expenses. No 401K matching has been budgeted for calendar 2005, although a limited matching program is planned for 2007 in an effort to keep 21st Century Medicine competitive with regard to labor requirements moving forward.

With the offsetting impact of funding other than LEF, mainly NIH grants, the overall net cost of research projects supported by LEF has been reduced with the allocation of overhead expenses spread over more than one source. As a result, the scope of research in these areas has increased without significant increases in LEF funding over the past three years.

Legal expenses for the application and administration of patents continue to increase, and the overall patent strategy merits review on an ongoing basis.

Insurance and maintenance expenses for the Edison Court building are expected to increase as we move into late 2006 and 2007.

12. Concluding Remarks

21CM has continued to create novel and cutting-edge research results and to push back the frontiers of biological preservation. In keeping with its past tradition, 21CM continues to tackle extremely challenging research projects that others have eschewed and, despite some inevitable setbacks, is succeeding in its research initiatives. At the same time, the company is developing stronger overtures from commercially significant entities and has more likelihood than ever of entering substantial commercial markets in the foreseeable future. Commercialization of technologies developed internally, or licensed from other organizations, remains a central goal and is the most important means of sustaining the long-term research goals of 21st Century Medicine and the Life Extension Foundation. In the meantime, the research funding received by 21st Century Medicine from the federal government beginning in 2002 has continued through 2006, and we are optimistic that this financial endorsement by the National Institutes of Health of 21CM research will continue.

The results of the sponsored projects, including all relevant information, will continue to be published in such form as to be available to the interested public either currently, as developments in the project warrant, or within a reasonably short time after completion of the project. If patent rights are involved, publication will take place after such rights are secured, as promptly as is reasonably possible.

21st Century Medicine, Inc. is very grateful for the past support of the Life Extension Foundation, and for our ongoing relationship. We take seriously our responsibility to maintain a research focus that is in the public interest, and to make information concerning our research available to the public as appropriate in the form of published articles, patents, and presentations.

21st Century Medicine will continue to report to the Life Extension Foundation how it has lived up to these ideals. The support of the Life Extension Foundation is critical to this important research, and we will continue to make our best efforts to achieve our scientific and commercial goals.

John Dean Barry, Chief Executive Officer

Gregory Fahy, Ph.D., Chief Scientific Officer and Vice President

Harvey Horswell, Chief Operating Officer

Brian Wowk, Ph. D., Senior Scientist

13. List of Appendices

A: Proposed operating budget for 2005
B: Reprints of papers published since the previous grant letter
C: Grant applications submitted to NIH since the previous grant letter
D: Patents awarded since the previous grant letter

21st Century Medicine
OPERATING BUDGET - FISCAL/CALENDAR 2006

PERSONNEL

1. Salaries/Taxes/Medical/Dental/Workman's Compensation Insurance

	Annual
Greg Fahy	155,000.00
John Dean Barry	170,000.00
Harvey Horswell	92,000.00
Brian Wowk	90,000.00
Jun Wu	96,000.00
Xian Ge	75,000.00
John Phan	43,000.00
Jesse Fuentes	40,000.00
Joon Chang	40,000.00
Laura Phan	20,000.00
Alice Chang	50,000.00
Yuangshen Tan	95,000.00
Lenetta Griffin	35,000.00
Overtime	
Total Gross Salary:	1,001,000.00

Administafff Service (% of Gross Payroll): 17.99%
Aggregate Annual pay increase: 0.00%

Total (annual): **$ 1,177,481.90**

2. 401K Plan/Auxillary Benefits

	401K Match	Insurance Buy Out		**Total**
Greg Fahy		0		0.00
John Dean Barry		0		0.00
Harvey Horswell		0		0.00
Brian Wowk		0		0.00
Jun Wu		0		0.00
Xian Ge		0		0.00
John Phan		0		0.00
Jesse Fuentes		0		0.00
Joon Chang		0		0.00
Patricia Wu		0		0.00
Alice Chang		0		0.00
Yuangshen Tan		0		0.00
Technician		0		
Totals:		0	**$**	-

3. Grand Total

Annual:	**$ 1,177,481.90**
Monthly:	**$ 98,123.49**

Doc. No. 0121

21CM Operating Budget, 2006
ADMINISTRATION

Non payroll related administration expense projections for fiscal 2006.

Account	Acct. #	Annual	Monthly
Education and Training	7115	5,000.00	416.67
Legal Fees	7121	100,000.00	8,333.33
Accounting Fees	7122	5,000.00	416.67
Consulting Fees	7123	5,000.00	416.67
Auto Insurance	7131		0.00
Facility Insurance	7132	24,000.00	2,000.00
Product Liability Insurance	7133	2,000.00	166.67
Environmental Insurance	7134		0.00
Building 1 Lease	7140	50,184.00	4,182.00
Business Travel	7150	20,000.00	1,666.67
Bank Fees	7161	500.00	41.67
Finance Costs	7162		0.00
Depreciation Expense	7163		0.00
Permits/Licenses	7171	1,000.00	83.33
Federal Tax	7172		0.00
State Tax	7173	800.00	66.67
County Tax	7174	10,000.00	833.33
Other Tax	7175	200.00	16.67
Office Supplies	7181	5,000.00	416.67
Computer Instal./repair	7182	8,000.00	666.67
Equipment repair/rental	7183	3,000.00	250.00
Postage, shipping	7184	4,000.00	333.33
Web Design/Service	7185	3,000.00	250.00
Employee Recruitment	7186	20,000.00	1,666.67
Miscellaneous	7199	5,000.00	416.67
CEO Housing	7151	18,000.00	1,500.00
Total		**$ 289,684.00**	**$ 24,140.33**

21CM Operating Budget, 2006
PHYSICAL PLANT EXPENSES

Account	Acct. #	Annual	Monthly
Security	7210	1200.00	100.00
Telephone	7221	9,000.00	750.00
Water	7222	1,800.00	150.00
Gas	7223	1,200.00	100.00
Electric	7224	30,000.00	2,500.00
Garbage	7231	2,300.00	191.67
Hazardous waste disposal	7232	5,000.00	416.67
Biohazardous waste disposal	7233	8,000.00	666.67
Other general maintenance	7240	15,000.00	1,250.00
Plant Equipment maintenance	7241	3,000.00	250.00
Equipment rental	7242	500.00	41.67
Landscaping	7243	2,100.00	175.00
Janitorial	7244	5,500.00	458.33
Pest Control	7245	900.00	75.00
Vehicle operation/repair	7250	0.00	0.00
Miscellaneous	7299	4,500.00	375.00
Total:		**$ 90,000.00**	**$ 7,500.00**

21CM Operating Budget, 2006
GENERAL LABORATORY EXPENSES

This expense category is for major laboratory equipment operations used by multiple research departments and/or projects. The expenses in this category will be allocated based on use to various research projects as required for financial analysis.

Account	Acct. #	Annual	Monthly
Blood Chemistry Analysis	7601	22,000.00	1,833.33
Glassware	7602	10,000.00	833.33
Inorganic (Na/K) Analysis	7603	1,500.00	125.00
Pipettors	7604	4,000.00	333.33
Electron Microscope	7605	10,000.00	833.33
Ciphergen Protein Chip System	7606	5,000.00	1,000.00
Laboratory Water System - Millipore	7607	4,000.00	333.33
Liquid Nitrogen	7608	10,000.00	833.33
HPLC	7609	7,000.00	583.33
Totals		**$ 73,500.00**	**$ 6,708.33**

21CM Operating Budget, 2006
CRYOBIOLOGY RESEARCH EXPENSES

Account	Acct. #	Annual	Monthly
Chemicals	7310	55,000.00	4,583.33
Supplies General	7330	35,000.00	2,916.67
Human Corneas	7331	0.00	0.00
Animal purchase	7351	40,000.00	3,333.33
Animal care	7352	4,000.00	333.33
Animal Medications	7354	7,000.00	583.33
Animal Surgical Supplies	7355	20,000.00	1,666.67
Contract research - ND	7361	0.00	0.00
Contract research - Mayo	7362	0.00	0.00
Contract research - Rochester	7363	0.00	0.00
Contract research/analytical	7379	20,000.00	1,666.67
Maintenance (cryo equip)	7380	2,000.00	166.67
Miscellaneous	7399	2,000.00	166.67
Total:		**$ 185,000.00**	**$ 15,416.67**

21CM Operating Budget, 2006
ICE BLOCKER RESEARCH EXPENSES

Account	Acct. #	Annual	Monthly
Chemicals - general	7410	5,000.00	416.67
Supplies	7430	5,000.00	416.67
Contract research/analytical	7450	0.00	0.00
Annealing research	7451	20,000.00	1,666.67
Maintenance	7460	2,000.00	166.67
Miscellaneous	7499	1,000.00	83.33
Total:		**$ 33,000.00**	**$ 2,750.00**

20CM Operating Budget, 2006
BRAIN CRYPRESERVATION RESEARCH EXPENSES

This budget category includes operating expenses only. It does not include personnel and capital equipment costs in support of the Brain Cryopreservation Project.

Account	Acct. #	Annual	Monthly
Perfusion machine materials	7811	5000.00	416.67
Animal Medication	7812	5000.00	416.67
Animal Care	7813	5000.00	416.67
Surgical supplies	7814	7000.00	583.33
Rabbits	7815	8400.00	700.00
Computer hardware and software	7816	3000.00	250.00
Non surgical Supplies	7817	10000.00	833.33
Chemicals	7818	10000.00	833.33
Microscopy and Histology	7822	5000.00	416.67
Maintenance	7821	2000.00	166.67
Miscellaneous	7823	2000.00	166.67
Total:		**$ 62,400.00**	**$ 5,200.00**

21CM Operating Budget, 2006
CHEMICAL PRODUCTS EXPENSES

Account	Acct. #	Annual	Monthly
Chemicals	7510	5000.00	416.67
Supplies	7530	3000.00	250.00
Maintenance	7550	1000.00	83.33
Shipping	7560	4000.00	333.33
Manufact. Misc.	7570	1000.00	83.33
Total:		**$ 14,000.00**	**$ 1,166.67**

21st CENTURY MEDICINE TOTAL FISCAL 2006 OPERATING BUDGET FOR LEF SPONSORED RESEARCH

The budget category totals listed represent the total funding required during 2006 assuming the research plan as detailed in the 2006 Grant Application Letter is adopted in full. Funds from sales and from money provided by NIH as "Administrative Overhead" have been applied in support of LEF sponsored research to offset the total cash requirement from the LEF.

Category	Annual	Monthly
Personnel	1,177,481.90	98,123.49
Administration	289,684.00	24,140.33
Physical Plant	90,000.00	7,500.00
Cryobiology Research	185,000.00	15,416.67
Ice Blocker Research	33,000.00	2,750.00
Brain Cryo Research	62,400.00	5,200.00
Chemical Products	14,000.00	1,166.67
General Laboratory	73,500.00	6,708.33
Capital Equipment	22,000.00	1,833.33
Total Proposed 2006 Operating Budget (Not including gov't sponsored research)	**$ 1,947,065.90**	**$ 162,255.49**
Sales and Gov't Grant Administrative Income	**$355,000.00**	**$29,583.33**
Total Operating Cash Requirement	**$1,592,065.90**	**$132,672.16**

Doc. No. 0129

21CM 2005 Federal Grant Applications

Form Approved Through 05/2004 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.

Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Extended Cardiac Preservation

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

New Investigator ☒ No ☐ Yes

3a. NAME *(Last, first, middle)*
Fahy, Gregory M.

3b. DEGREE(S)
B.S. Ph.D

3c. POSITION TITLE
Chief Scientific Officer

3d. MAILING ADDRESS *(Street, city, state, zip code)*
21st Century Medicine, Inc.
10844 Edison Court
Rancho Cucamonga, CA 91730

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Cryobiology Research

3f. MAJOR SUBDIVISION

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (909) 466-8633 FAX: (909) 466-8618

E-MAIL ADDRESS: gfahy@21cm.com

4. HUMAN SUBJECTS RESEARCH
☒ No
☐ Yes

4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No.

4b. Human Subjects Assurance No.

4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date

5b. Animal welfare assurance no

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD	8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
7/01/04	06/30/06	$583,000	$790,639	$957,587

7b. Total Costs ($): $699,320

9. APPLICANT ORGANIZATION
Name 21st Century Medicine, Inc.
Address 10844 Edison Court
Rancho Cucamonga, CA 91730

Institutional Profile File Number (If known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☒ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
33-0559567
DUNS NO. *(if available)*
Congressional District 42

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Harvey Horswell
Title Chief Operating Officer
Address 10844 Edison Court
Rancho Cucamonga, CA 91730

Tel (909) 466-8633 FAX (909) 466-8618
E-Mail hhorswell@21cm.com

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Harvey Horswell
Title Chief Operating Officer
Address 10844 Edison Court
Rancho Cucamonga, CA 91730

Tel (909) 466-8633 FAX (909) 466-8618
E-Mail hhorswell@21cm.com

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* DATE 12/12/03

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* DATE 12/12/03

Doc. No. 0131

LEF000146

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The long-term objective of this project is the commercialization of a product(s) that enables a substantial improvement in human heart preservation prior to transplantation. The ability to significantly extend human clinical cardiac preservation, to 24 hours or more, would allow transcontinental prospective matching, with expected gain in clinical outcome for potentially thousands of transplant recipients. Phase I studies provided a proof-of-concept for the feasibility of an automated intermittent perfusion device, that used in conjunction with the preservation solution (URS) described in Phase I, successfully preserved canine hearts for at least 24 hours. Data from phase I also demonstrated that the use of URS alone, not in combination with intermittent perfusion, results in substantial extension of cardiac preservation when compared to current commercial preservation formulations. Based on these findings, it is now our strategy to introduce two products sequentially: first, URS as a stand alone product for cardiac preservation, and later, an intermittent perfusion device that will build on the first product release and offer yet further extension of cardiac preservation. In phase II we plan to demonstrate the utility of URS for 24 hour storage using post-transplant survival in animal models as an end point, and to investigate the limits of URS for the preservation of human hearts. It is our intention to use this body of data as evidence to persuade the FDA to grant clearance for 21st Century Medicine to proceed with multicenter, randomized human clinical trials to begin phase III. Additionally, in phase II, the development of the intermittent perfusion device as a powerful preservation modality will continue. Ultimately the device may become a second generation product as currently envisioned. However, it is also possible the data from human heart studies in phase II will demonstrate that intermittent perfusion with URS is indeed necessary to achieve the desired extension of human cardiac preservation. Should that be the case, the combination product, URS with an intermittent perfusion system, will be tested with greater priority.

PERFORMANCE SITE(S) *(organization, city, state)*

21st Century Medicine, Inc., Rancho Cucamonga, CA

Behavior Sciences Foundation, St. Kitts, Caribbean

University of Rochester, Rochester, NY

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Fahy, Gregory	21st Century Medicine, Inc.	Principal Investigator
Wowk, Brian	21st Century Medicine, Inc.	Project Engineer
Massey, Todd	University of Rochester	Transplant Surgeon
Wang, Tingchung	University of Rochester	Collaborating Investigator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes **X No**

Principal Investigator/Program Director (Last, first, middle): Fahy, Gregory M.

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT

TABLE OF CONTENTS

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Check if Appendix is Included ☐

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list):

Doc. No. 0133

LEF000148

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 07/01/04 THROUGH 06/30/05

PERSONNEL (Applicant organization only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Fahy, Gregory M.	Principal Investigator	12	10.0				17,400
Wowk, Brian	Engineering	12	17.0				16,320
To be named	Machinist	12	12.5				6,250
To be named	Cardiophysiologist	12	40.0				16,800
SUBTOTALS →							56,770

CONSULTANT COSTS

EQUIPMENT *(Itemize)*
Electronic/computer equipment, supporting software: $19,600. Pumps, pacers, defibruillator: $5,800. Incubator: $6,000. Electromagnetic flowmeter: $20,000. 51,400

SUPPLIES *(Itemize by category)*
Transducers, oxygenators, sensors: $4,400. Chemicals/perfusate prep: $23,150. Electronic assembly components: $4,500. Glassware, filters, tubing, bags: $4,020. Non-human primates: $1,500. Human hearts: $4,400. Safety: $3,000. Blood cells, filters, processing supplies: $15,300. 87,270

TRAVEL
UR staff to 21st Century Medicine, 21CM staff to Primate Products, FedEx shipments 4,500

PATIENT CARE COSTS — INPATIENT

OUTPATIENT

ALTERATIONS AND RENOVATIONS *(Itemize by category)*

OTHER EXPENSES *(Itemize by category)*
Space at 21st Century Medicine to accommodate blood perfusion testing equipment: $45,000. Testing, histology, electron microscopy, tissue and blood analysis: $4,250. 49,250

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$ 249,190
CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	218,148
	FACILITIES AND ADMINISTRATIVE COSTS	125,435
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)* →		$ 592,773
SBIR/STTR Only: FEE REQUESTED		10,000

Doc. No. 0134

LEF000149

Program Director (Last, First, Middle): Fahy, Gregory M.

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: Salary and fringe benefits. Applicant organization only.		56,770	69,139			
CONSULTANT COSTS		0				
EQUIPMENT		51,400	4,500			
SUPPLIES		87,270	109,500			
TRAVEL		4,500	7,500			
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		49,250	17,000			
SUBTOTAL DIRECT COSTS		249,190	207,639			
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	218,148	0			
	F&A	125,435	0			
TOTAL DIRECT COSTS		592,773	207,639			

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)*						$ 800,412
SBIR/STTR Only Fee Requested		10,000	10,000			20,000
SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period (Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.)						$ 10,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Dr. Fahy, as Principle Investigator, will oversee and coordinate the entire project. Dr. Wowk will assist with the construction of the blood perfusion machine in Year 1 and the upgrades to the CardioStat in Year 1 and Year 2. The machinist will be hired on a part time basis to allow fabrication of components of the blood perfusion machine and of the CardioStat. The Cardiophysiologist will be required full time to receive human hearts from OPOs 24 hours a day, 7 days a week, to prepare and restock all required prefusates at 21st Century Medicine and at cooperating OPOs who accept URS from our laboratory, to prepare the blood perfusion machine for use and to clean and disinfect it after experiments, to keep and process accurate experimental records, to physically prepare and manipulate human and non-human primate hearts using universal precautions, and to evaluate all hearts tested using the refurbished IP device.

No equipment needed for the blood perfusion machine is presently available. The specific items quoted include numerous smaller items.

H&E section and 1 EM sample will be examined per each of 25 human and 5 non-human primate hearts, at per light microscopy slide and $200 per EM workup (total = $6,450). ATP determinations will cost $4,300. Blood analyses will be done at the beginning, middle, and end of each blood perfusion for 30 hearts at a cost of $150 per sample. The total of these costs will be $21,250, of which 20% will be expended in Year 1 and 80% in Year 2.

The supplies required include sensors needed for cardiac evaluation in vitro, perfusate ingredients for solutions that will be required independently of the number of hearts received in order to ensure the presence of fresh solutions on very short notice (KHB at $20/liter, URS at $100/liter, and washed red cells at $200/liter), and our initial 5 human hearts at $880 each. Special safety precautions are required (gowns, gloves, masks, caps, eye shields, cold sterilant for non-autoclavable components, disinfectants, etc. at $100/experiment x 30 experiments). Non-human primate costs are based on fees for animals, services, and supplies as quoted by Primate Products, Inc.

Travel of Dr. Wang to Florida will be required to ensure proper cardiac acquisition methods are taught to Primate Products, Inc. staff ($850 complete cost). Travel of Dr. Wang to 21st Century Medicine is desired to assist us with the initial two monkey heart evaluations ($1000 complete cost) and with the setup of the apparatus ($1000). Non-human primate heart shipping via Federal Express with sufficient wet ice will cost $100 for each of 7 hearts. In year 2, we request travel for Dr. Wang and Dr. Fahy to present our results at one scientific meeting each at $1,500 per meeting and for 21st Century Medicine CEO J. Dean Barry to attend trade shows to promote our technology ($4,500).

Direct costs of upgrading floor plan to accommodate cardiophysiology testing equipment is estimated at $3,000 based on local contractor rates.

Year 2 salaries reflect employment of the cardiophysiologist for the entire year rather than for a partial year in Year 1, this cost being partly offset by reduced time requirements on the part of Dr. Wowk. The increase in supplies and other costs reflects a dramatic increase in the number of human hearts being processed in Year 2. Equipment needs are greatly reduced in Year 2 since most equipment needed for the project will have been purchased by the end of Year 1, even if it is not used until Year 2.

Principal Investigator/Program Director (Last, first, middle): Fahy, Gregory M.

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 7/01/04 THROUGH 6/30/05

PERSONNEL (Applicant organization only) NAME	ROLE ON PROJECT	TYPE APPT. (months)	% EFFORT ON PROJ.	INST. BASE SALARY	DOLLAR AMOUNT REQUESTED (omit cents) SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Fahy, Gregory M.	Principal Investigator	12	10				17,400
Wowk, Brian	Engineering	12	17				16,320
To be named	Machinist	12	12.5				6,250
To be named	Cardiophysiologist	12	40				16,800
SUBTOTALS →							56,770

CONSULTANT COSTS

EQUIPMENT (Itemize)
Electronic/computer equipment, supporting software: $19,600. Pumps, pacers, defibrillator: $5,800. Incubator: $6000. Electromagnetic flowmeter: $20,000. 51,400

SUPPLIES (Itemize by category)
Transducers, oxygenators, sensors: $4,400. Chemicals/perfusate prep: $23,150.
Electronic assembly components: $4,500. Glassware, filters, tubing, bags: $4,020.
~~Vervets: $17,877.~~ Human hearts: $4,400. Safety: $3,000.
Blood cells, filters, processing supplies: $15,300.
$ 28,500 76,647

TRAVEL
UR staff to 21st Century Medicine, 21CM staff to St. Kitts, FedEx shipments 5,350

PATIENT CARE COSTS — INPATIENT
OUTPATIENT

ALTERATIONS AND RENOVATIONS (Itemize by category)

OTHER EXPENSES (Itemize by category)
~~Build-out of existing~~ space at 21st to accommodate ~~blood~~ perfusion ~~laboratory:~~ $45,000. (for testing equipment)
Testing, histology, electron microscopy, tissue and blood analysis: $4,250. 49,250

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$239,417
CONSORTIUM/CONTRACTUAL COSTS Univ. of Rochester	DIRECT COSTS	218148
	FACILITIES AND ADMINISTRATIVE COSTS	125435
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (Item 7a, Face Page) →		$583,000
SBIR/STTR Only: FEE REQUESTED		10,000

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Doc. No. 0137

LEF000152

Principal Investigator/Program Director (Last, first, middle): Fahy, Gregory M.

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS		INITIAL BUDGET PERIOD (from Form Page 4)	ADDITIONAL YEARS OF SUPPORT REQUESTED 2nd	3rd	4th	5th
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*		56,770	69,139			
CONSULTANT COSTS						
EQUIPMENT		51,400	4,500			
SUPPLIES		76,647	109,500			
TRAVEL		5,350	7,500			
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
OTHER EXPENSES		49,250	17,000			
SUBTOTAL DIRECT COSTS		239,417	207,639			
CONSORTIUM/ CONTRACTUAL COSTS	DIRECT	218,148	0			
	F&A	125,435	0			
TOTAL DIRECT COSTS		583,000	207,639			

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD *(Item 8a, Face Page)* — **$ 790,639**

SBIR/STTR Only Fee Requested	10,000	10,000			

SBIR/STTR Only: Total Fee Requested for Entire Proposed Project Period
(Add Total Fee amount to "Total direct costs for entire proposed project period" above and Total F&A/Indirect costs from Checklist Form Page, and enter these as "Costs Requested for Proposed Period of Support on Face Page, Item 8b.) — **$ 20,000**

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Dr. Fahy, as Principle Investigator, will oversee and coordinate the entire project. Dr. Wowk will assist with the construction of the blood perfusion machine in Year 1 and the upgrades to the CardioStat in Year 1 and Year 2. The machinist will be hired on a part time basis to allow fabrication of components of the blood perfusion machine and of the CardioStat. The Cardiophysiologist will be required full time to receive human hearts from OPOs 24 hours a day, 7 days a week, to prepare and restock all required perfusates at 21st Century Medicine and at cooperating OPOs who accept URS from our laboratory, to prepare the blood perfusion machine for use and to clean and disinfect it after experiments, to keep and process accurate experimental records, to physically prepare and manipulate human and vervet hearts using universal precautions, and to evaluate all hearts tested using the refurbished IP device.

No equipment needed for the blood perfusion machine is presently available. The specific items quoted include numerous smaller items.

One H&E section and 1 EM sample will be examined per each of 25 human and 5 vervet hearts, at $15 per light microscopy slide and $200 per EM workup (total = $6450). ATP determinations will cost $4300. Blood analyses will be done at the beginning, middle, and end of each blood perfusion for 30 hearts at a cost of $150 per sample. The total of these costs will be $21,250, of which 20% will be expended in Year 1 and 80% in Year 2.

The supplies required include sensors needed for cardiac evaluation in vitro, perfusate ingredients for solutions that will be required independently of the number of hearts received in order to ensure the presence of fresh solutions on very short notice (KHB at $20/liter, URS at $100/liter, and washed red cells at $200/liter), and our initial 5 human hearts at $880 each. Special safety precautions are required (gowns, gloves, masks, caps, eye shields, cold sterilant for non-autoclavable components, disinfectants, etc. at $100/experiment x 30 experiments). ~~Vervet~~ costs are based on ~~cost of~~ animals, services, and supplies as quoted by ~~St. Kitts.~~ PRIMATE PRODUCTS

Travel of ~~Dr. Fahy to St. Kitts~~ will be required to ensure proper cardiac acquisition methods are taught to ~~St. Kitts~~ staff (~~$1200 air fare, $100 hotel stay, $50 car rental~~). Travel of Dr. Wang to 21st Century Medicine is desired to assist us with the initial two monkey heart evaluations ($1000 complete cost) and with the setup of the apparatus ($1000). ~~Vervet~~ heart shipping via Federal Express with sufficient wet ice will cost ~~$200~~ for each of 7 hearts. In Year 2, we request travel for Dr. Wang and Dr. Fahy to present our results at one scientific meeting each at $1,500 per meeting and for 21st Century Medicine CEO J. Dean Barry to attend trade shows to promote our technology ($4,500).

Direct costs of ~~constructing the cardiophysiology laboratory are~~ estimated at $15,000 based on local contractor rates. ~~An additional $10,000 is needed to equip the room with lab benches, sinks, eye wash stations, etc.~~

Year two salaries reflect employment of the cardiophysiologist for the entire year rather than for a partial year in Year 1, this cost being partly offset by reduced time requirements on the part of Dr. Wowk. The increase in supplies and other costs reflects a dramatic increase in the number of human hearts being processed in Year 2. Equipment needs are greatly reduced in Year 2 since most equipment needed for the project will have been purchased by the end of Year 1, even if it is not used until Year 2.

BIOGRAPHICAL SKETCH AND BIBLIOGRAPHY

Gregory M. Fahy, Ph.D. Chief Scientific Officer and Director of Research
Twenty-First Century Medicine, Inc.

Education

Institution and Location	Degrees	Date Conferred	Field of Study
University of California, Irvine	B.S.	1972	Biology
Medical College of Georgia, Augusta	Ph.D.	1993	Pharmacology/Cryobiology

A. Professional Experience/Honors:

Positions and Employment:

1998-date Vice President and Chief Scientific Officer, Twenty-First Century Medicine, Inc. Directs intramural R&D pertaining to vitrification of cells, tissues, and whole mammalian organs, organ cold storage, and cryopreservation by freezing, including research on antinucleation, advanced cryoprotectants, novel perfusates, and novel perfusion devices for treating organs with cryoprotectants. Supervises one MD/Ph.D. surgeon, one Ph.D. pathologist, one Ph.D. physical chemist, one Ph.D. neurobiologist, four laboratory technologists, and one animal care technologist. Invented several new generations of advanced cryoprotectant and cryoprotectant introduction and removal techniques, culminating in the preservation solution and protocol to be studied in more detail in this proposal. Established current and past extramural cooperative research projects with Genzyme Biosurgery, the Mayo Clinic, the University of Rochester, the Cedars-Sinai Medical Center, the University of Wisconsin, Solvay Pharmaceuticals, the University of Wales College of Medicine, and other institutions to develop preservation methods for cartilage, corneas, hearts, livers, kidneys, organ slices, ova, and sperm. Filed 8 patent applications in the US and abroad, including two issued patents. Obtained awards of over $310,000 in current SBIR grants to 21st Century Medicine.

1995-1997 Head, Tissue Cryopreservation Section, Naval Medical Research Institute, Chief Scientist, Organ, Inc., and Chief Scientist, LRT, Inc. Directed a team of three Ph.D.s, one engineer, and three technologists. Created the concept of synthetic "ice blockers," discovered a means for eliminating "chilling injury" at -20°C and below in organs and tissues, discovered ways of protecting vascular endothelium during the perfusion of organs with vitrification solution, published the first paper showing immediate function of kidneys after perfusion with vitrifiable concentrations of cryoprotectant, developed electromagnetic warming equipment and organ blood perfusion equipment. Submitted well over 10 patent applications, the vast majority of which have issued. Responsible for award of $1.9 million NIST grant.

1981-1995 Scientist II and Project Leader, American Red Cross. Introduced the concept of organ and tissue vitrification and the first practical means of attaining this goal. Created the first useful vitrification solution (VS1) and published first proof of principle of vitrification using VS1 in *Nature* with collaborator, using mouse embryos as a model. Led team of three investigators, one engineer, and two technologists devoted to developing successful organ cryopreservation methods. Invented, built, programmed, used, and published first automated equipment for perfusing whole organs with cryoprotectant. Submitted multiple patent applications, all of which issued.

1977-1980 Postdoctoral Fellow ('77-'79) and Research Associate, American Red Cross. Developed analytical techniques for understanding freezing injury in living systems and explored liquid state preservation at subzero temperatures as low as –80°C. Began development of RPS-2, a long-term cold storage solution for kidneys. Conceived the idea of vitrification of organs.

Professional Activities and Awards:

Grand Prize for Medicine, 1995, International Patent Exposition (INPEX), for organ cryoprotectant perfusion machine. Treasurer, Society for Cryobiology, 1985-1987. Symposia/workshop editor for Cryobiology, 1986 and 1989. Society for Cryobiology contact person for devices. Consultant, U.S. Pharmacopeial Convention, 1993-1994. Referee for 8 journals and 4 organizations, including NIH. Member of editorial board of Journal of Cell Preservation Technology and Journal of Anti-Aging Medicine. Presented over 60 invited seminars and symposium presentations, including invited symposium or keynote talks at the last three consecutive Society for Cryobiology meetings with another invitation for 2004. Over 120 scientific publications.

B. Most Pertinent Publications and Patents:

Fahy, G.M., and Hirsh, A. Prospects for organ preservation by vitrification. In: Organ Preservation, Basic and Applied Aspects (D.E. Pegg, I.A. Jacobsen, and N.A. Halasz, eds.), MTP Press, Lancaster, 1983, pp. 399-404.

Fahy, G.M. Cryoprotectant toxicity neutralizers reduce freezing damage. Cryo-Letters 4: 309-314, 1983.

Fahy, G.M. Cryoprotectant toxicity: biochemical or osmotic? Cryo-Letters 5: 79-90, 1984.

Fahy, G.M. Cryoprotectant toxicity reduction: specific or nonspecific? Cryo-Letters 5: 287-294, 1984.

Fahy, G.M., MacFarlane, D.R., Angell, C.A., and Meryman, H.T. Vitrification as an approach to cryopreservation. Cryobiology 21: 407-426, 1984.

Rall, W.F., and Fahy, G.M. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. Nature 313: 573-575, 1985.

Fahy, G.M. Vitrification: a new approach to organ cryopreservation. Prog. Clin. Biol. Res. 224: 305-335, 1986.

Fahy, G.M. The relevance of cryoprotectant 'toxicity' to cryobiology. Cryobiology 23: 1-13, 1986.

Fahy, G.M., Levy, D.I., and Ali, S.E. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology 24: 196-213, 1987.

Fahy, G.M. Vitrification. In: Low Temperature Biotechnology, Engineering Contributions (J. McGrath and K.R. Diller, Eds), ASME, pp. 113-146, 1988.

Fahy, G.M., Lilly, T.H., Linsdell, H., St. John Douglas, M., and Meryman, H.T. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology 27: 247-268, 1990.

Fahy, G.M., Saur, J., and Williams, R.J. Physical problems with the vitrification of large biological systems. Cryobiology 27: 492-510, 1990.

Fahy, G. M. Organ perfusion equipment for the introduction and removal of cryoprotectants. Biomed. Instr. Technol. 28: 87-100, 1994.

Khirabadi, B.S., Fahy, G.M., Ewing, L., Saur, J., and Meryman, H.T. 100% survival of rabbit kidneys chilled to -32°C after perfusion with 8M cryoprotectant at -22°C. Cryobiology 31: 597, 1994.

Fahy, G.M., and Ali, S.E. Cryopreservation of the mammalian kidney. II. Demonstration of immediate ex vivo function after introduction and removal of 7.5 M cryoprotectant. Cryobiology 35: 114-131, 1997.

Khirabadi, B.S., and Fahy, G.M. Permanent life support by kidneys perfused with a vitrifiable (7.5 molar) cryoprotectant solution. Transplantation 70: 51-57, 2000.

Wowk, B., and Fahy, G.M. Inhibition of bacterial Ice nucleation by polyglycerol polymers. Cryobiology 44: 14-23, 2002.

Fahy, G.M., and Wowk, B. Cryoprotectant solution containing dimethyl sulfoxide, an amide, and ethylene glycol. US Patent 6,395,467 B1, May 28, 2002.

Fahy, G.M., and Wowk, B. Prevention of ice nucleation by polyglycerol. US Patent 6,616,858, Sept. 9, 2003.

Fahy, G.M., Wowk, B., Wu, J., and Paynter, S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 44: (in press), 2004.

Fahy, G.M., Wowk, B., Wu, J., Phan, J., Chang, A., and Zendejas, E. Cryopreservation of organs by vitrification: perspectives and recent advances. Cryobiology 44: (in final review), 2004.

C. Research Support.

Ongoing Research Support:

Fahy (PI) 01/01/02-12/31/04
Life Extension Foundation Award
Cryopreservation of Neuronal Tissue.
The purpose of this study is to develop means of cryopreservation of neuronal tissue to aid in the effort of neuropyschiatric drug discovery.
Role: PI

Fahy (PI) 04/01/03-03/31/04
NIH 1 R43 DK063806-01
Improved Renal Preservation
The purpose of this study is to investigate new protocols to enable substantial improvements in outcomes after extended periods of kidney storage.
Role: PI

Fahy (PI) 08/15/02-07/31/03 (extended to 07/31/04)
NIH 1 R43 HL66813-01A1
Extended Cardiac Preservation
The purpose of this study is to investigate means of extending viable storage times for heart explants by means of improved storage solutions and intermittent perfusion storage protocols.
Role: PI

Principal Investigator/Program Director (Last, first, middle): Fahy, Gregory M.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Brian Wowk, Ph.D.	Senior Physicist 21st Century Medicine, Inc.

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Manitoba, Canada	B.Sc.	1990	Physics
University of Manitoba, Canada	M.Sc.	1993	Medical Physics
University of Manitoba, Canada	Ph.D.	1997	Medical Physics

Professional Experience

1997-date Senior Physicist, 21st Century Medicine, Inc.
Current responsibilities include developing novel compounds that inhibit ice growth by non-colligative mechanisms ("ice blockers"), engineering of organ perfusion systems, and solving both scientific and engineering problems of storing tissue cryopreserved by vitrification. Experience includes design and construction of portable systems for cardiac perfusion, and construction of three different types of cryogenic storage units for vitrified materials.

993-1997 Graduate Student, National Research Council of Canada
Ph.D. on magnetic resonance imaging of human brain function (fMRI).

1991-1993 Graduate Student, Manitoba Cancer Treatment and Research Foundation

1991-1992 Research Assistant, Manitoba Cancer Treatment and Research Foundation

1991 Teaching Assistant, University of Manitoba Physics Department

1990 Summer Student, Manitoba Cancer Treatment and Research Foundation

Publications and Patents

B. Wowk, G.M. Fahy, "Inhibition of bacterial ice nucleation by polyglycerol polymers," Cryobiology 44, 14-23 (2002).

S.B. Harris, M.G. Darwin, S.R. Russell, J.M. O'Farrell, M. Fletcher, B. Wowk, "Rapid (0.5C/min) minimally invasive induction of hypothermia using cold perfluorochemical lung lavage in dogs," Resuscitation 50 189-204 (2001).

B. Wowk, E. Leitl, C.M. Rasch, N. Mesba-Karimi, S.B. Harris, G.M. Fahy, "*Vitrification enhancement by synthetic ice blocking agents*," Cryobiology 40, 228-236 (2000).

B. Wowk, M. Darwin, S.B. Harris, S.R. Russell, C.M. Rasch, "*Effects of Solute Methoxylation on Glass-Forming Ability and Stability of Vitrification Solutions*," Cryobiology 39, 215-227 (1999).

B. Wowk, M.C. McIntyre, J.K. Saunders, "*k-space Detection and Correction of Physiological Artifacts in fMRI*," Magnetic Resonance in Medicine 38, 1029-1034 (1997).

B. Wowk, S. Shalev, "*Thick phosphor screens for on-line portal imaging*," Medical Physics 21, 1269-1276 (1994).

B. Wowk, T. Radcliffe, K.W. Leszczynski, S. Shalev, R. Rajapakshe "*Optimization of metal/phosphor screens for on-line portal imaging*," Medical Physics 21, 227-235 (1994).

B. Wowk, T. Radcliffe, S. Shalev, "*Grooved phosphor screens for on-line portal imaging*," Medical Physics 20, 1641-1651 (1993).

T. Radcliffe, G. Barnea, B. Wowk, R. Rajapakshe, S. Shalev, "*Monte Carlo optimization of metal/phosphor screens at megavoltage energies*," Medical Physics 20, 1161-1169 (1993).

"Prevention of ice nucleation by polyglycerol" U.S. Patent #6,616,858. Inventors: G. Fahy, B. Wowk. 2003.

"Cryoprotectant solution containing dimethyl sulfoxide, an amide and ethylene glycol" U.S. Patent #6,395,467. Inventors: G. Fahy, B. Wowk. 2002.

"Polyvinyl alcohol compounds for inhibition of ice growth" U.S. Patent #6,391,224. Inventors: B. Wowk. 2002.

"Method for Rapid Cooling and Warming of Biological Materials" U.S. Patent #6,274,303. Inventors: B. Wowk, M.G. Federowicz, S.R. Russell, S.B. Harris. 2001.

"Method for Vitrification of Biological Materials Using Alkoxylated Compounds" U.S. Patent #5,952,168. Inventors: B. Wowk, M.G. Federowicz, S.R. Russell, S.B. Harris. 1999.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Howard Todd Massey, MD	**Assistant Professor**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Georgia, Athens, Georgia	BS	1982-1986	Biochemistry
Medical College of Georgia, Augusta, Georgia	MD	1986-1990	Medicine

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. **Positions and Honors.** List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

1990-1995	General surgery resident, University of Louisville, Louisville, Kentucky
1995-1998	Thoracic & cardiovascular surgery resident, University of Texas, Southwestern, Dallas, TX
1998-2000	Adult cardiac surgery fellow, Heart and Lung Transplantation, Duke University Medical Center, Durham, NC
9/2000 - date	Assistant Professor, University of Rochester Medical Center, Rochester, NY
9/2000 - date	Chair, Lung Transplantation Task Force, University of Rochester Medical Center, Strong Memorial Hospital
9/2000 – date	Surgical Director, Program in Heart Failure and Transplantation, University of Rochester Medical Center, Strong Memorial Hospital

B. **Selected peer-reviewed publications (in chronological order).** Do not include publications submitted or in preparation.

1. Hicks GL and Massey HT 2002. Update on indications for surgery in aortic insuffiency. Current Opinion in Cardiol 17:172-178.

C. **Research Support.** List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

1. Long-term cardiac preservation in dogs – PI Dr. Tingchung Wang.
   Role: collaborating investigator.
2. Phase II Pilot Study to Compare the Efficacy of Octacol F15 with Standard of Care Topical Hemostats in the Time to Achieve Hemostasis After Primary or Re-do Coronary Arterial Bypass Grafting (CABG) Procedures
   Role: PI
3. Quintiles CABG Study - PI Dr. Delehanty
   Role: collaborating investigator.
4. Thoratec's AEGIS/US Clinical Trial for the atrial coronary artery bypass graft
   Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Wang, Tingchung	Research Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
National Taiwan University, Taiwan	BS	1966	Botany
University of Minnesota, St. Paul, MN	PhD	1974	Cell Biology

A. **Positions and Honors.** List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

1974-1979 Research Associate, Department of Surgery, University of Minnesota
1979-1982 Assistant Professor, Department of Surgery, University of Minnesota
1982-1984 Assistant Professor, Department of Surgery, Tulane University
1984-1987 Associate Professor, Department of Surgery, Tulane University
1987 - 1996 Senior Scientist, Department of Surgery, University of Rochester
1996 - date Research Associate Professor, Dept. of Surgery, University of Rochester

B. **Selected peer-reviewed publications (in chronological order).** Do not include publications submitted or in preparation.

1. Vulapalli SR, Chen Z, Chua B, Wang T, Liang CS. Cardioselective Overexpression of HO-1 Prevents Ischemia-Reperfusion Induced Cardiac Dysfunction and Apoptosis. Am. J. Physiol. Heart Cir Physiol. 283, 688-694, 2002.
2. Takeishi Y, Huang Q, Wang T, Glassman M, Yoshizumi M, Lee J, Kawakatsu H, Baines C, Che W, Ohta S, Walsh R, Berk B, Abe J. Src family kinase and adenosine differentially regulate multiple MAP kinases in ischemic myocardium: Modulation by Ischemic Preconditioning. J. Mol. Cell. Cardiol. 33, 1989-2005, 2001.
3. Samuel P, Zhang X, Yoshizumi M, Snider J, Risher, WH, Berk, B, Hicks GL, Wang T. Aprotinin pretreatment improves hypothermic cardiac preservation involving bradykinin receptors and protein kinase C. Surg Forum 50:103-105, 1999.
4. Ward H. Baldwin D, Wang T, et al. Ion-exchange column chromatographic method for assaying purine metabolic pathway enzymes. J Chromatography B 707:295-300, 1998.
5. Afifi H, Zhang C, Risher WH, Hicks GL, Wang T. Donor pretreatment with adenosine A1 agonist enhances long-term preservation of the cardiac explant. Surgical Forum 48: 267-270, 1997.
6. Andreacchi AS, Wang T, Wu JHD. Cardiovascular effect of the fungal extraction of Basiiomycetes sp. YL8006. Life Sci 60, 1987-1994, 1997.
7. Zhu Q,Yu W, Yang X, Hicks GL, Lanzafame JR, Wang T. Photo-irradiation improved functional preservation of the isolated rat heart. Lasers Surg. Med. 19: 1996.
8. Yang X, Zhu Q, Fong J, Gu X, Hicks GL, Bishop S, Wang T. Enalaprilat, an angiotensin-converting enzyme inhibitor, enhances functional preservation during long-term cardiac preservation. Possible involvemnet of bradykinin and PKC. J. Mol. Cell. Cardiol. 28, 1445-1452, 1996.
9. Zhu Q, Yang X, Claydon MA, Hicks GL, Wang T. Twenty-four hour intermittent perfusion storage of the isolated rat heart: II. Effect of perfusion pressure on functional preservation. J.Surg. Res. 61, 159-164, 1996.
10. Mugnano JA, Wang T, Layne JR, DeVries AL, Lee R. Antifreeze glycoproteins promote intracellular freezing of rat cardiomyocytes at high subzero temperatures. Am. J. Physiol. 269, R474-79, 1995.
11. Costanzo JP, Lee RE, DeVries AL, Wang T, Layne JR. Survival mechanisms of vertebrate ectotherms at subfreezing temperatures: application in cryomedicine. FASEB J. 9, 351-358, 1995.
12. Zhu Q, Yang X, Claydon MA, Hicks GL, Wang T. Twenty-four hour intermittent perfusion storage of the isolated rat heart: The effect of perfusion intervals on functional preservation. J. Heart Lung Transpl. 13, 882-890, 1994.
13. Watanabe BI, Hicks GL, Wang T. Deferoxamine treatment during reperfusion but not during storage improves function of the hypothermically stored rat heart. J. Surg. Res. 57, 164-169, 1994.

14. Yang X, Zhu Q, Claydon MA, Hicks GL, Wang T. Enhanced functinal preservation of cold-stored rat heart by a nucleoside transport inhibitor. Transplantation 58, 28-34, 1994.
15. Wang T, Zhu Q, Yang X, DeVries AL, Layne JR. Antifreeze glycoproteins from antartic notothenioid fishes do not protect the cardiac explant during freezing and non-freezing preservation. Cryobiology 31, 185-192, 1994.
16. Zhu Q, Yang X, Claydon MA, Hicks GL, Wang T. Adenosine deaminase inhibitor in cardioplegia enhanced function preservation of the hypothermically-stored rat heart. Transplantation 57, 35-40, 1994.
17. Yang X, Zhu Q, Layne JR, Claydon M, Hicks GL, Wang T. Subzero nonfreezing storage of the mammalian cardiac explant. I. Methanol, ethanol, ethylene glycol, and propylene glycol as colligative cryoprotectants. Cryobiology, 30, 366-375, 1993.
18. Banker MC, Hicks GL, Wang T. Multi-dose cardioplegia as a strategy for twenty-four hour functional and metabolic preservation of the cardiac explant. Surg. Forum 43, 246-248, 1992.
19. Zhu Q, Layne JR, Claydon M, Hicks GL, Wang T. Freezing preservation of the mammalian cardiac explant. VII. Effect of thawing rate on functional recovery. Cryobiology 29, 478-484, 1992.
20. Wang T, Banker MC, Claydon M, Hicks GL, Layne JR. Freezing preservation of the mammalian cardiac explant. VI. Cryopreservation by ethanol. Cryobiology 29, 470-477, 1992.
21. Banker MC, Layne JR, Hicks GL, Wang T. Freezing preservation of the mammalian cardiac explant. III. Tissue dehydration and cryoprotection by polyethylene glycol. J. Heart and Lung Transplantation. 11, 619-623, 1992.
22. Banker MC, Layne JR, Hicks GL, Wang T. Freezing preservation of the mammalian cardiac explant. II. Comparing the protective effect of glycerol and polyethylene glycol. Cryobiology 29, 87-94, 1992.
23. Banker MC, Layne JR, Hicks GL, Wang T. Freezing preservation of the mammalian cardiac explant. IV. Functional recovery after 8-hour freezing. Curr Surg. 48: 428-430, 1991.
24. Banker MC, Hicks GL, Wang T. Evidence that stimulation of adenosine A1-receptors enhances preservation of the cardiac explant during long-term hypothermic storage. Surg. Forum 42: 299-301, 1991.
25. Wang T, Connery CP, Batty PR, Hicks GL, DeWeese JA, Layne JR. Freezing preservation of adult mammalian heart at high subzero temperatures. Cryobiology 28, 171-176, 1991.
26. Wang T, Batty PR, Hicks GL, and DeWeese JA. Long-term hypothermic storage of the cardiac explant. Comparison of four solutions. J. Cardiovasc. Surg. 32: 21-25, 1991.
27. Connery CP, Hicks GL, Wang T. Positive correlation of functional recovery and tissue ATP levels in the hypothermically-stored cardiac explant. Surg. Forum 41:282-284, 1990.
28. Batty PR, Hicks GL, DeWeese JA, and Wang T. The osmolality requirement of the cardiac explant during cold storage. J. Surg. Res. 48:601-605, 1990.
29. Wang T, Tan Z, Webb WR: Postnatal changes in enzyme activities of rat myocardial adenine nucleotide catabolic pathway. Life Sci. 40: 239-244, 1987.
30. Ward, Einzig S, Wang T, Bianco RW, Borgwardt B, Marquardt C, Foker JE:Comparison of catecholamine effects on myocardial metabolism, cardiac performance and regional blood flow following myocardial ischemia on cardiopulmonary bypass. J. Thorac.Cardiovasc. Surg. 87:452-4654, 1984.
31. Ward H, Wang T, Einzig S, Bianco RW, Foker JE: Prevention of ATP catabolism during myocardial ischemia: A preliminary report. J Surg. Res. 34:292-297, 1983.
32. Ward HB, Wang T, Einzig S, Bianco RW, Foker JE: Consequences of acute B-adrenergic blockade on myocardial ischemia during cardiopulmonary bypass. Curr. Surg. 40:106-114, 1983.
33. Ward HB, Wang T, Einzig S, Bianco RW, Foker JE:Enhanced myocardial ATP recovery following ischemia with enzyme inhibition and precursor infusion. J. Am. Coll. Cardiol. 1:666, 1983.
34. Ward HB, Einzig S, Wang T, Bianco RW, Foker JE: Enhanced cardiac efficiency with dobutamine after global ischemia. J. Surg. Res. 33:32-38, 1982.
35. Ward HB, Einzig S, Bianco RW, Wang T, Foker JE: Effects of dopamine and dobutamine on the myocardial and systemic circulation during and following cardiopulmonary bypass in dogs. Ped Cardiol. 3:257-264, 1982.
36. Ward HB, Wang T, Einzig S, Bianco RW, Foker JE: Enhanced regeneration of postischemic myocardial adenosine triphosphate concentrations. Surg. Forum 32:254, 1981
37. Foker JE, Einzig S, Wang T: Adenosine metabolism and myocardial preservation: The consequences of adenosine catabolism on myocardial high energy compounds and tissue blood flow. J. Thorac. Cardiovasc. Surg. 80:506, 1980.
38. Batty PR, Hicks GL, DeWeese JA, and Wang T. The calcium requirement for hypothermic storage of the cardiac explant. Curr. Surg. 46:313-316, 1989.

C. **Research Support.** List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and your role (e.g. PI, Co-Investigator, Consultant) in the research project. Do not list award amounts or percent effort in projects.

Ongoing research

1. 1R43HL66813-01A1 NIH/NIHBL G. Fahy (PI) 8/15/2002 – 7/31/2003.
"Extended Cardiac Preservation" This SBIR phase 1 grant to 21st Century Medicine, Inc, Rancho Cucamonga, CA is to develop an automated cardiac preservation pump system.
Role: As a project leader of a contract from 21st Century Medicine, Inc, to test the pump using orthotopic cardiac transplantation dog model.

2. 50845T, AHA, New York Affliliated 7/2000-6/2004
"Long-term cardiac preservation by intermittent perfusion". The project is to study high energy phosphate metabolism and tissue perfusion in dog heart during hypothermic storage.
Role : P.I.

3. Rochster Eye and Human Parts Bank 4/2000-7/2003
"Cornea Preservation". The project is to study the preservation of endothelium of human cornea in oxygen-enriched environment.
Role: P.I.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:
21st Century Medicine has 4000 sq. ft. of fully equipped laboratory including: protemoics lab, electron microscopy lab, cell culture lab, wet lab, perfusion lab, and complete rabbit surgical facilities. All facilities are at the same location and fully available for this project. The Behavior Sciences Foundation of St. Kitts operates a full primate laboratory including surgical suites for anesthesia, sedation, and euthanasia of monkeys. It also has lab facilities suitable for ocular examination by clinical specular microscopy. The University of Rochester has analytical instrumentation available for data acquisition and analysis. Also available is a fully equipped cardiology animal surgery suite and associated instrumentation.

Clinical:

Animal:
21st Century Medicine has adjacent to the labs a complete rabbit vivarium, USDA licensed and overseen by its internal IACUC committee. The Behavioral Sciences Foundation (BSF) maintains a colony of 800+ vervets, and all related surgical procedures are performed on site. BSF has a full health maintenance and monitoring program in place, is OLAW compliant and holds various NIH and other federal agency grants and contracts. The University of Rochester has fully available a 1000 sq. ft. physiological and biochemical lab in its Division of Cardiology. An animal operating room is available. The vivarium is directed by an ACLAM board certified veterinarian and staff.

Computer:
All major lab equipment, as well as all personal computer workstations, at 21st Century Medicine are linked through a common network. The company uses only up-to-date computer equipment and software. Both the University of Rochester and the Behavioral Sciences Foundation have computer facilities expected for a major approved research center.

Office:
All key personnel on this project, as well as administrative personnel have full office facilities at both locations.

Other:
21st Century Medicine has a full maintenance and electronics shop, warehouse, shipping and loading facilities.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

21st Century Medicine: Electron micsoscope, Ciphergen mass spectrometer proteomics system, light microscopes, fluorescence microscope, full animal surgical suite and supporting facilities, autoclaves, sterilization and water purification systems, electrophoresis equipment, wet lab and hoods, centrifuges, balances, glassware, refrigerators and freezers. All equipment is in the same building. Key to this project is equipment for: preparation, storage and transport of solutions, sterilization of apparatus, storage device manufacture.

Behavioral Sciences Foundation: Full primate surgical suites and care facilities, with associated laboratories.

University of Rochester: Full canine surgical suites and care facilities, with associated laboratories.

RESEARCH PLAN

A. SPECIFIC AIMS

The objective of the proposed research is to provide strong evidence for the feasibility of dramatic extension of the permissible duration of hypothermic storage of human hearts in preparation for potential human clinical trials of the University of Rochester solution (URS) in Phase III. It is clear that flushing with URS is able to permit simple cold storage of the canine heart for at least 24 hours based on acute functional tests, but this result must be confirmed by permanent transplantation and life support before human clinical trials of the solution can begin. It would also be desirable to obtain evidence for the efficacy of the solution in a non-human primate model and to develop methods for testing URS-preserved human hearts in vitro to ensure that human hearts can adequately function after being preserved by the methods envisioned for use in human clinical trials. Finally, the promise of "intermittent perfusion" (IP), which refers to the periodic interruption of static cold storage with controlled bouts of perfusion at defined times, should continue to be developed for clinical use in view of the extraordinary duration of preservation it is capable of achieving and in view of its ability to resuscitate damaged hearts, a feature that might ultimately lead to an increase in organ supply. In view of these pressing needs, we propose the following specific aims.

1. Demonstrate permanent life support after 24-hour simple cold storage of the canine heart Dog hearts will be preserved by flushing them with URS and holding them at 0°C for 24 hours without IP followed by orthotopic transplantation. The recipients will be allowed to survive for 3 months during which the function of the grafts and the well being of the recipients will be evaluated.

2. Develop a blood perfusion system for cardiac functional evaluation and use it to demonstrate functional recovery of primate hearts after 24-hour simple cold storage. A blood perfusion device will be constructed according to previous designs and used to test the function of monkey (vervet) hearts flushed for us with URS in St. Kitts and shipped to us by Federal Express for evaluation the following day.

Primate Products ?

3. Demonstrate the functional recovery of human hearts after 24-hour storage. Using the device proven out in Aim 2, we will evaluate human hearts obtained from organ procurement organizations and stored for a total period of 24 hours from the time of initial procurement. Hearts will be procured after initial cardioplegia with either URS or UW solution and will be subsequently stored in URS, UW solution, or Celsior, in some cases after perfusion at 25°C (IP) to reverse injury sustained up to that time and establish a common functional baseline for subsequent solution comparisons.

4. Refine the IP device. We will implement a number of very advantageous design changes to improve all aspects of the attractiveness and functionality of the IP device. The improved design will be tested using human hearts first as physical models and then with subsequent functional evaluation by blood perfusion.

B. BACKGROUND AND SIGNIFICANCE

Background. We have now demonstrated, in a convincing model system, the unequivocal feasibility of preserving large mammalian hearts for 24 hours using two different methods (simple cold storage and IP). This has not been reported before in the literature. For over three decades various experimental procedures claimed long-term preservation (> 24 h) of the animal hearts (1-6). Notwithstanding, static hypothermic immersion, which offers 4 to 6 hours of storage time, remains the only method used clinically. This remarkably short storage time severely limits the availability of donor hearts, increases costs, and creates risks for patients. Measures applied as possible means for increasing the current time limit for cardiac storage have included: continuous perfusion (7, 8), the maintenance of "high energy phosphates" or HEP (9,

10), the removal of metabolic byproducts (11), the provision of additional oxygen using perfluorocarbon (12, 13), and metabolic (14)(17) or pharmacological (15) supplementation. Cardiac preservation for 24 hours has been claimed using the Na^+/H^+ exchange inhibitor Hoe 642 (15), Nicorandil (15), adenosine (17), dibutyryl cAMP and nitroglycerin (18), nucleoside transport blockade (19), and coenzyme Q_{10} (20). However, all of these studies used clinically ambiguous methods of assessing the functionality of the preserved heart. Although it is valid for research on mechanisms of cardioprotection to use methods that do not demand full functional recovery of a stored heart, this is not the case for work intended to develop convincing cardiac preservation techniques for actual clinical application. For the latter purpose, it is imperative to use a large animal orthotopic cardiac transplant model to assess the efficacy of the storage method. Because of this requirement, a large portion of past observations on cardiac preservation has little direct clinical relevance, and in fact almost none of the findings that went beyond 24 hours showed successful preservation of actual cardiac function. So far, despite a remarkable report in 1968 claiming 72 hour storage (3), no reliable long-term method has ever been established. Therefore, our demonstration of complete recovery of donor heart function in a canine orthotopic cardiac transplant model after 40 (67) up to 49 hours of storage was a significant milestone. The following phenomena are believed to be key factors in our success.

HEP and cardiac preservation - Conventionally, cardiac preservation is achieved by metabolic arrest and energy conservation using chemical cardioplegia and hypothermia. This was based on the observations that myocardial ATP levels are intimately related to postischemic cardiac viability and function (21-24). If myocardial ATP content is reduced below a threshold level during ischemia, function will not recover after reperfusion (25). In the case of heart preservation, as soon as a donor heart is excised, global ischemia is induced. Without tissue perfusion or an oxygen supply, metabolism quickly reverts to anaerobic glycolysis. Energy expenditure soon exceeds production causing the HEP reserve to decline. With both cardioplegia and hypothermia, myocardial metabolism as assessed by oxygen consumption can be reduced to less than 2% of normal for a beating heart (26). Nevertheless, the residual metabolic rate is still high enough to exhaust the energy reserve in a few hours. Moreover, the accumulation of metabolic wastes and the development of tissue acidosis inhibit glycolysis, thus further exacerbating the problem of energy imbalance. During early ischemia, myocardial ATP levels are maintained at the expense of phosphocreatine (PCr). When PCr levels are depleted to a level that can no longer keep ATP levels constant, ATP decreases. Depletion of HEP has been proposed as the most likely factor limiting the duration of hypothermic immersion storage (27), and our proposed research ultimately originates from evidence that preservation of myocardial HEP, particularly including PCr and ATP, successfully leads to extended cardiac preservation (see Preliminary Results, below).

Perfusion-induced tissue edema - Perfusion supplies O_2 and nutrients and eliminates the accumulation of potentially toxic metabolic byproducts. It should be the preferred method for long-term cardiac preservation. However, perfusion of the heart with crystalloid perfusate induces myocardial edema (28-30). Cell swelling caused by water uptake renders the heart stiff and reduces myocardial compliance, decreases left ventricular volume, and generally impairs function (30-32). Different species vary in the susceptibility of their hearts to perfusion-induced edema (30-32). The so-called microperfusion technique, which involves continuous low-flow perfusion, does not induce edema but suffers from an inability to overcome the critical closing pressure of myocardial capillaries, and therefore to attain uniform distribution of perfusate throughout the heart. We too encountered the edema problem when we perfused the dog heart continuously even when we used a very low perfusion rate in combination with colloid osmotic support from 6% hydroxyethyl starch. The problem, however, was not present when we stored the heart using our IP method.

Intermittent perfusion (IP) – Hypothermic multidose cardioplegia has long been used routinely during open heart surgery. There are also studies of prolonged cardiac preservation using multidose cardioplegia or IP. Agrawall et al (33) found that IP for 2 min every 6 hours preserved the viability of the isolated rat heart during 24-hour storage. Tani and Neely (34) investigated the protective mechanisms of IP in the isolated rat heart. IP attenuated both the increase in intracellular sodium during ischemia and the rise in calcium during reperfusion. Segel and Follette (35) studied the preservation of the isolated rat heart with cycles of 5 or 10 min of perfusion followed by 25 min of no perfusion. Post-storage function of IP hearts after 12 hours of storage was significantly better than that in hearts stored by immersion. Several studies showed the

advantages of IP on myocardial adenine nucleotide and phosphocreatine status during or after hypothermic storage. Dyszkiewicz et al. (36) stored the dog heart for 24 hours at 0.5°C with IP at 4, 8, and 12 hours. They found that myocardial ATP content was better preserved by IP. Lockett et al (37) studied in rabbit earts the status of HEP using ^{31}P magnetic resonance spectroscopy (MRS). The hearts were stored for 24 hours then reperfused with 6-8°C cardioplegic solution. A 30 min cold reperfusion restored myocardial phosphocreatine levels to normal. In an orthotopic canine heart transplantation model, Ohtaki et al (38) reported that myocardial HEP levels and tissue histology were greatly restored by 60 min of cold cardioplegic reperfusion following 12 hours of cold immersion storage. Significantly better functional performance was observed in hearts that received post-storage reperfusion. The latter two studies showed that cardioplegic perfusion effectively restored myocardial HEP and function. The latter two studies showed that cardioplegic reperfusion can be used effectively to restore myocardial HEP. Although proposed mechanisms by which IP may work (39) (40)(34) remain speculative, the success of IP is a documented fact. We believe it is an ethical obligation to do what we can to ultimately deliver the benefits of this technology and the benefits of simple cold storage using URS to patients in need.

Very recently, Organ Recovery Systems, Inc. (ORS) has obtained FDA approval for the marketing and use of a perfusion machine developed for the preservation of kidneys, and has plans to introduce a similar machine for the preservation of hearts. We believe the efforts of this group are not truly competitive to the proposed research for two reasons. First, as reviewed above, continuous perfusion is inappropriate for the preservation of hearts due to the peculiar sensitivity of the heart to the formation of edema, and the unusually significant effect of edema in this organ. The main alternative to continuous perfusion, i.e., simple cold storage, does not require their device, and we believe there is no solution that approaches the efficacy of URS for simple cold storage. Second, their device is not capable of preserving hearts or other organs by IP because of several incompatibilities between their machine design and the requirements of IP, the most obvious of which is that their machine achieves control over organ temperature only as a result of continuous perfusion.

The purpose of ORS's devices is to evaluate organ function prior to transplantation to enable viable rgans and non-viable organs to be identified as such prior to being either transplanted or discarded. This oncept was originated by the Principle Investigator, whose group constructed a blood perfusion machine that was successfully used to evaluate the function of rabbit kidneys in vitro before the initiation of ORS's efforts (68,69). The current ORS device is designed to evaluate kidneys damaged by extensive warm ischemia and is not capable of evaluating ordinary preserved hearts. It is unlikely that any ORS device for the functional evaluation of human hearts at physiological temperatures will become commercially available within the period covered by the proposed research. However, the Principle Investigator's experience with normothermic renal blood perfusion and 21CM staff's experience with the construction of automated perfusion devices will make it readily possible for us to construct our own blood perfusion system for the evaluation of human hearts under conditions that adequately mimic the in vivo environment.

Significance. The ability to transport hearts over transcontinental or even intercontinental distances for transplantation or for research evaluation would revolutionize the field of human cardiac transplantation and positively affect the 2,500 recipients of human heart transplants every year in the United States, many heart transplant patients overseas, and many additional patients who currently need heart transplants but are be unable to receive them as a result of residing outside the geographical range of available donor hearts created by the present 4-hour cardiac storage limit. In addition to enabling long distance transportation of human hearts, extended cardiac preservation would result in better quality donor hearts, better graft and patient survival, huge improvements over the present frenetic logistics of arranging transplants on a non-elective basis, large reductions in cost, and eventually even prospective cross matching of normal or pre-immunized patients, which is known to have the ability to positively influence long-term transplant outcomes. In addition, although URS has been tailored to preservation of the heart per se, our IP technique may be applicable to other short-lived organs such as the lung (safe current storage time limit, ~6 hours) (63, 64), the pancreas (~15-20 hours) (65), and liver (~15-20 hours) (65), independently of the utility of URS for these organs. This could expand the significance of the proposed research to the approximately 18,000 non-ardiac transplants performed in the US each year and a roughly equal number in the rest of the world,

particularly if IP can be shown to be either biologically, economically, or psychologically superior to continuous perfusion for the preservation of these organs, which is very possible. Further, our use of normothermic blood perfusion as a means of assessing the value of organ preservation methods intended for clinical use by enabling direct studies on human organs may provide a beneficial model for others to follow in future studies. This model maximizes the clinical meaningfulness of experimental results by eliminating species differences between man and animals as a confounding factor while also minimizing animal use. It is also possible that our use of warm perfusion to resuscitate hearts damaged by prior cold storage will ultimately lead to the salvage of more human hearts for transplantation, thereby increasing the supply of donor organs and saving additional lives.

C . PRELIMINARY STUDIES

The development of URS. Unlike the development of other preservation solutions for the heart, URS has been developed as a result of systematic study at UR over a number of years (44) to determine the optimum concentrations of calcium (45, 46), 2,3-butanedione monoxime (47), and potassium and magnesium (48), as well as the optimum pH and type of pH buffer (49, 50), and osmolality (46, 51) for cardiac preservation by simple cold storage. URS, also known as CP-11EB, has been successful after 22-26 hours of simple cold storage. In this experiment, the dog hearts were arrested with CP-11EB, and stored on ice for 22 to 26 h, and then transplanted orthotopically into the recipients. All six recipients were successfully weaned off cardiopulmonary bypass. As can be seen in Table 1, the hemodynamic performance of the grafted hearts 6 hours after weaning off bypass was excellent. Confirmatory evidence was also obtained from studies of the effects of IP on URS-preserved hearts. These studies showed that after at least 20 hours of storage at 0°C, all deleterious changes in energy metabolism (HEP levels) and tissue pH were completely reversible, and subsequent decay rates were not different from rates of change observed with the storage of fresh hearts (see next section).

Table 1 Successful preservation of donor hearts for 24 hours using URS without IP

Experiment No.	1	2	3	4	5	6	Mean±SE
Storage Hours	22	23	23	24	25	26	23.8±1.5 h
SAP (mm Hg)*	125	125	100	120	100	100	112±13
DAP (mm Hg)*	70	75	75	60	60	60	67±8

* SAP, systolic arterial pressure and DAP, diastolic arterial pressure 6 hours after ending CPB

These results appear superior to those attainable with the two leading cardiac preservation solutions on the market. Dog hearts stored with UW solution, the leading general organ preservation solution, showed a 72% decline in mitochondrial respiration that made restoration of adequate contractility unlikely after 24 hour storage (52), and a microperfusion study with UW solution claimed a storage time limit of only 12 hours (54). Although Celsior has been introduced recently specifically for cardiac preservation, only 2 of 6 dog hearts stored in Celsior for 24 hours could be weaned from cardiopulmonary bypass, and even when continuous perfusion was used, only 4 of 6 hearts preserved for 24 hours could be weaned from bypass (53). Takeuchi et al. (55) used a blood perfusion model to show that a histidine-containing cardioplegia solution preserved functional capacity (stroke volume, ejection fraction, developed pressure) of dog hearts for 24 hours much better than those stored in UW solution. But these results have not yet been confirmed by orthotopic cardiac transplantation.

The development of IP. Much more effort has been devoted historically to the development of ultra-long storage periods using IP. We initially found that IP prolonged the storage time of the isolated rat heart. The hypothesis that IP may act in part by repleting myocardial energy stores led to a number of interesting observations. Using brief IP at 25°C to minimize perfusion-induced edema, IP quickly repleted ATP after both 10 and 17 hr of storage at 0°C (41). Hearts stored with no perfusion at 0°C for 24 hours failed to recover function after reperfusion. In comparison, those receiving only two 2-minute IPs (at 10 and 17 hours of

storage) recovered 50% of pre-storage function. More frequent perfusion with intervals shorter than 10 hours was undesirable (42). In a third study, 60 and 70 mm Hg pressure provided the best preservation of cardiac function (43). More recently, in unpublished studies, we applied IP to the preservation of dog hearts, five of which were cardioplegically arrested and stored by immersion at 0°C for 40 h. At 20 and 36 hours of storage, the heart was perfused with a 25°C cardioplegic solution (CP-11EB) at a perfusion pressure of 55 mm Hg for **5 min**. At 40 hr, the heart was orthotopically transplanted into a recipient. After the graft resumed rhythmic sinus beating, the recipient was weaned off cardiopulmonary bypass (CPB). Hemodynamic and contractile function was normal, and remained stable without inotropic support for 6 hours after discontinuing CPB (**Table 2**). IP induced negligible edema during storage, heart wet weight (175±20 g) increasing by only 3±1 g (1.4%) after perfusion at 20 hours and by 10±2 g (5.8%) after perfusion at 36 h. In contrast, hearts stored for 40 hours without IP did not develop normal rhythm and could not support the recipient off bypass.

Table 2 Functional indices of canine hearts preserved for 40 hours, orthotopically transplanted, and weaned from cardiopulmonary bypass

Hours off CPB	heart rate (beats/min)	Systolic pressure (mm Hg)	Diastolic pressure (mm Hg)	+dP/dt (mm Hg/sec)	-dP/dt (-mm Hg/sec)
1	145±20	105±22	42±5	986±204	-684±350
2	129±12	102±19	46±7	1035±297	-503±208
4	96±9	109±6	48±9	1134±246	-458±106
6	96±4	111±6	53±8	1031±152	-350±52

Figure 1 shows representative ^{31}P-MR spectra of one of 3 dog hearts that were cardioplegically arrested in situ and stored at 0°C. At time zero, there were 3 large ATP peaks and a very prominent PCr peak. After 18 hours of cold storage, ATP peaks were broader but shorter, indicating some ATP loss, and PCr practically disappeared from the spectrum. After just 5 min of 25°C cardioplegic perfusion (18 hours + IP), a small PCr peak appeared, and ATP peaks became larger. The leftmost peak is inorganic phosphate (Pi), which was shifted to the right after storage, indicating intracellular acidosis. IP shortened this Pi peak. ATP and PCr decreased further between 18 and 36 hours, while the Pi peak became significantly larger. Again, with just 5 min of IP (36 hours + IP), PCr reappeared, ATP peaks increased, and the Pi peak was attenuated. Thus IP "recharged" PCr and ATP in the cold stored heart, reduced the levels of Pi, and reversed acidosis.

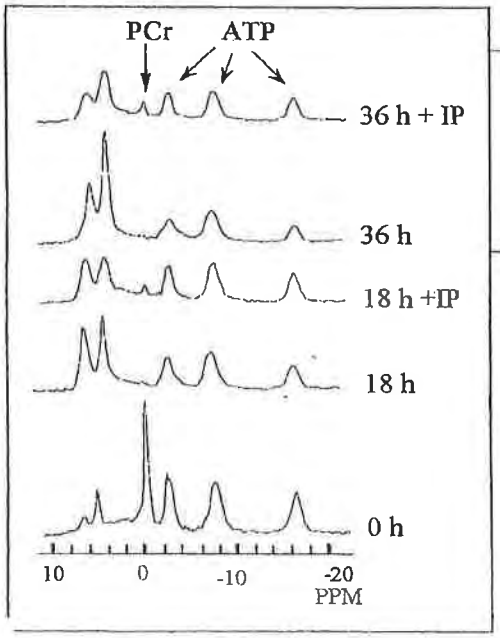


Fig. 1 (Left): ^{31}P-MRS of dog heart at different cold storage times before and after (+IP) intermittent perfusion .

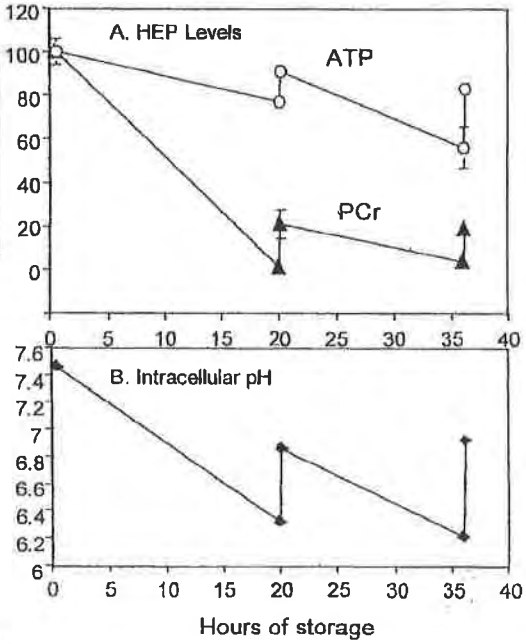


Figure 2 (Right): IP reversal of ATP, PCr, and pH declines after 20 and 36 hrs of cold storage in canine hearts. HEP levels expressed as percent of values at zero time. Means ± SD.

Similar data normalized to time zero values are shown as a function of time in **Figure 2.** PCr fell to 1% of the pre-storage level at 20 hr, but rebounded to 21% after 5 min of IP. ATP decreased by 23% at 20 hr, but rebounded to 91% with IP. Intracellular pH fell to 6.3 at 20 hours and was partially restored after IP. Similar declines and rebounds were observed after an additional 16 hr of storage and IP at 36 hours.

We also investigated the IP duration required to effect desired changes in cardiac temperature and pH by following pulmonary artery temperature as a good indicator of tissue temperature and coronary effluent pH as an indicator of tissue pH **(Figure 3).** Two dog hearts were cardioplegically arrested and stored at 0°C for 10 hours then perfused at room temperature with oxygenated perfusate. A thermocouple probe inserted into the pulmonary artery was connected to a telethermometer to measure temperature. Coronary effluent was collected and pH was determined every 5 min. Pulmonary artery temperature rose rapidly with perfusion and reached perfusate temperature in around 10 min. In contrast, coronary effluent pH did not change very much for the first 5 min but then increased quickly to a plateau pH of 7.5 at around 20 min of perfusion. These results corroborated an earlier ^{31}P -MRS observation that intracellular pH reached a plateau only after 20-25 min of perfusion. Based on this experiment, later studies employed an IP duration of 20 min or greater.

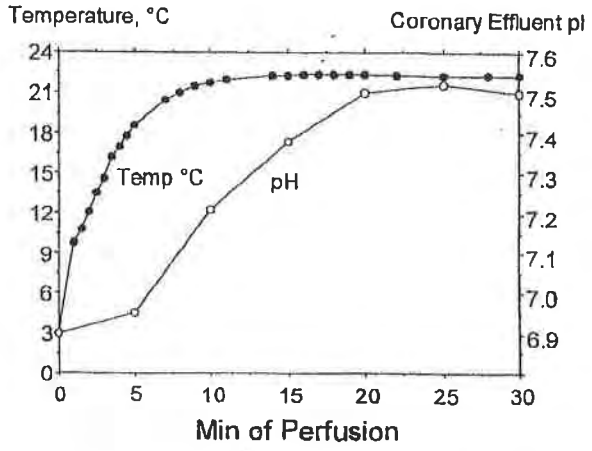


Figure 3: Pulmonary arterial temperature and coronary effluent pH during IP.

In order to better evaluate the effects of 20 min IP, more extensive baseline (no IP) left ventricular values of HEP, Pi, and pH were first obtained from drill biopsies from 4 dog hearts after arrest and storage at 0°C (**Figure 4**;data normalized to time zero ^{31}P-MRS-derived values). Myocardial PCr levels decreased rapidly to 23±2% of baseline by 7 hours of storage and to 8±1% by 24 hours. ATP levels fell to 44±3% of initial values at 24 hours, and Pi increased 3.7 fold by 7 hours, remaining relatively steady thereafter. Intracellular pH was 7.6±0.2 at time zero and declined and diverged into 2 prominent and persistent pH domains within 3 hours. At 24 hours, the pH values for the two domains were 6.4 and 6.2.

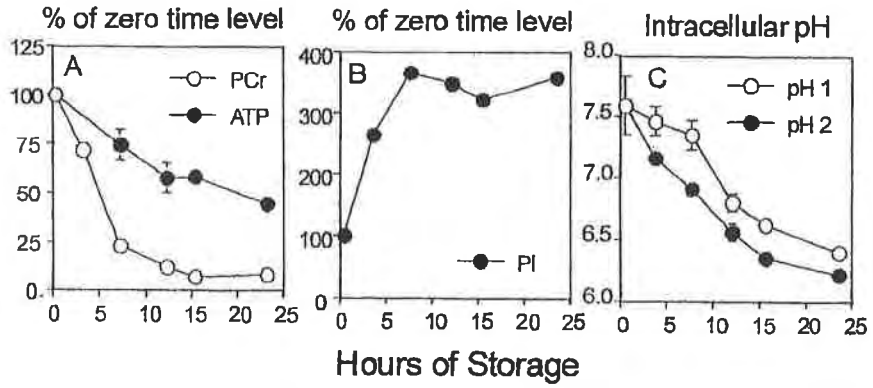


Fig. 4: Changes in myocardial PCr, ATP, inorganic phosphate, and intracellular pH during cold storage of the dog heart. Panel A: PCr and ATP; panel B: inorganic phosphate; panel C: intracellular pH. All data were expressed as a percentage of zero time values. Means ± 1 SE.

In **Figure 5**, we show the effects of 20 min of IP and the kinetics of HEP depletion following this IP. Dog hearts (n=4) were cardioplegically arrested and stored by immersion in 0°C URS for 11 hours. The hearts were then perfused with oxygenated 22°C URS for 20 min. Biopsies of the right ventricles were taken

at time zero (for normalization of subsequent data) and immediately before IP at 11 hours of storage. Biopsies of the left ventricle were taken immediately after IP and then several times over an additional 13 hours of 0°C storage following IP. All biopsies were immediately subjected to ^{31}P- MRS. The closed points in Figure 5 recapitulate the baseline data from Figure 4 for comparison, and the open points show events immediately before IP and subsequent to IP.

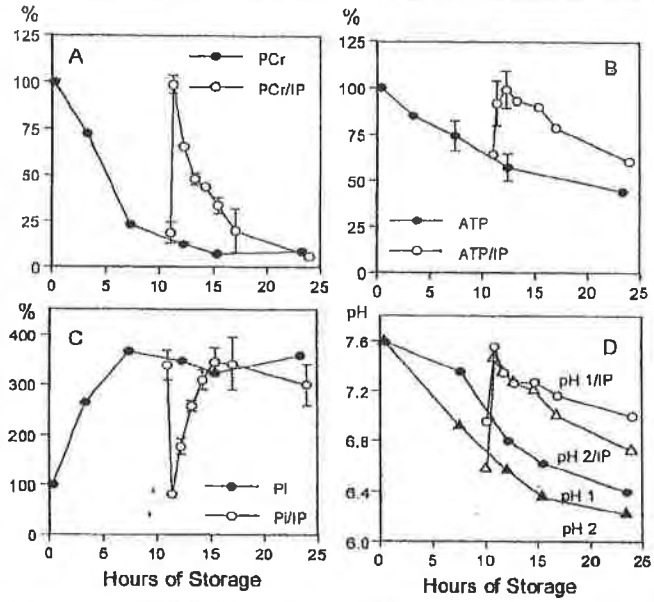
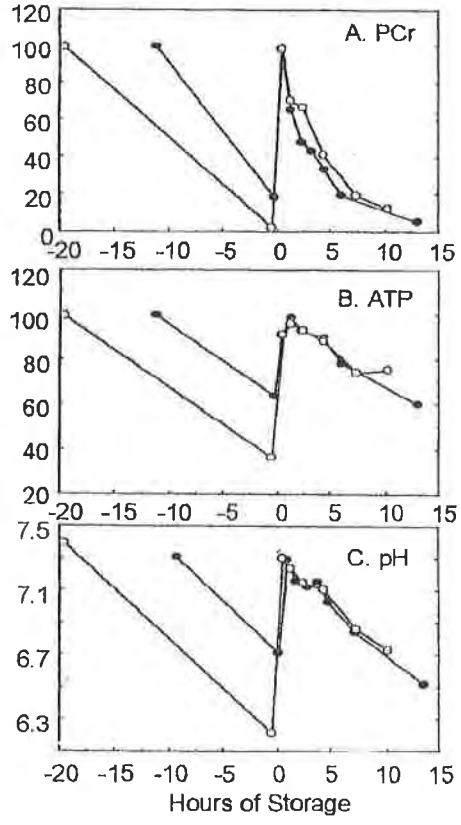


Figure 5 (Left). For discussion, see text.

The results show that after IP, both PCr and ATP were restored fully to time zero levels (open circles). Similarly, Pi (Fig. 5C) was reduced from 340% of baseline levels at 11 hours to 81% of baseline levels after IP. Both intracellular pH domains were also completely restored to the time zero value after IP. It therefore appeared that 20 min of IP was indeed a sufficient time for HEP repletion and pH correction according to these results, in agreement with the suggestion from the simple measurements of Figure 3. In addition, the post-IP decay rates of HEP and pH further suggested that the reversal of storage injury by IP might be nearly complete in that the kinetics of HEP and pH decline after IP were very similar to the kinetics of these processes prior to IP.



Effect of IP on dog hearts cold-stored for 20 hours In Table 2 we showed the effects of 5 min IP at 20 hours of cold storage. To verify and extend these results, we performed 20 min IP at 20 hours of storage in 4 dog hearts and followed HEP, Pi, and pH before and after IP. **Figure 6** shows the 20 hour, 20 min IP data in comparison to the 11 hour, 20 min IP data of Figure 5. Remarkably, the two data sets are superimposable when synchronized to the time of onset of IP (20 hour data = closed circles; 11 hour data = open circles). Therefore, **even after cold storage for as long as 20 hours, myocardial HEP metabolism was completely restorable to a status reflective of zero storage time.** In other words, the entire time course of

Fig. 6 (Left): Changes in PCr, ATP, and pH in hearts receiving IP at 20 hours of cold storage (open circles) or at 11 hours (closed circles). The time of IP was plotted as a new time zero in order to compare repletion and decay rates after IP at the two different storage times. As can be seen, the final magnitudes after both repletion and subsequent decay were identical, suggesting that 20 hours does not approach the limit of 0°C URS cold storage beyond which damage reversal by IP is incomplete.

HEP depletion and intracellular acidosis seen normally starting from time zero and continuing onward can be shifted to the right by at least 20 hours. This observation truly demonstrates the essence of our IP method – prolongation of storage by delaying the depletion of HEP and decline in intracellular pH, as if the zero time of storage has been reset, "wiping clean" the injury that has since been accumulated.

In summary, IP for 20 min at 11 and 20 hours of cold storage completely repleted PCr and ATP levels, and normalized Pi and intracellular pH to pre-storage values, and even restored the kinetics of HEP depletion, Pi rise, and reduction in intracellular pH back to the rates observed starting from time zero.

The two intracellular pH domains of Fig. 4C presumably indicate the existence of two metabolically distinct regions of myocardium, a phenomenon seen before in normal and ischemic myocardium (57). These could reflect the subepicardium and the subendomyocardium, or they could reflect cytosolic and mitochondrial pHs (58). Regardless of their identity, we were able to correlate myocardial ATP levels with both pH domains, ATP levels decreasing as intracellular pH fell (**Figure 7**). This suggests that ATP synthetic capability and/or ATP consumption was intracellular pH-dependent in the cold-stored heart. In light of our hypothesis that maintenance of HEP is crucial to cardiac preservation, interventions to maintain physiological intracellular pH may be beneficial to myocardial HEP metabolism and in turn enhance cardiac preservation. Based on these important findings we carried out a new series of transplantation experiments whose results are listed in the next section.

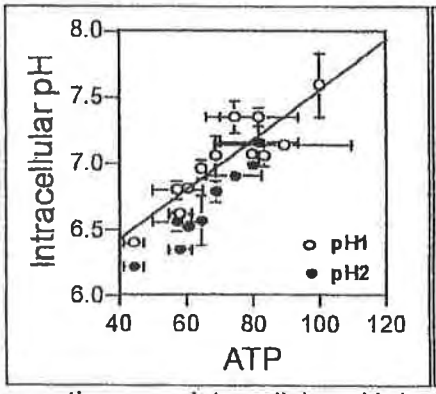


Figure 7 (left): correlation between intracellular pH and ATP content (as a % of initial ATP levels). Overall regression line is shown. Separate significant correlations also exist for each domain individually.

Successful dog heart preservation for 40-49 hours. Based on the findings above, we administered IP bouts of 20-40 min so as to completely replete HEP during a 40-49 hour preservation period and submitted the hearts to orthotopic transplantation as usual. The mean systolic and diastolic pressures for 5 historical controls preserved for 39-40 hours were 111±6 and 53±8 mm Hg, respectively. The results of preservation for over 40 hours as shown in **Table 3** below compare favorably to these historical results, and there was no difficulty in weaning the recipients of the 43.5 – 49-hour hearts from cardiopulmonary bypass.

Table 3. Successful Preservation for 40-49 hours Using IP with Extended IP Bouts

Preservation Time (hrs)	40	41	43.5	45.5	46.5	47.5	49
Systolic Pressure (mm Hg)*	115	100	103	115	104	120	142
Diastolic Pressure (mm Hg)*	58	65	54	50	69	65	78
Ejection fraction (EF)*	60%	40%	60%	50%	57%	60%	65%

*Pressure and EF maintained by the transplanted heart 6 hrs after implantation

The best results in Table 3 were obtained with the heart that was preserved the longest, to 49 hrs. **Since there was no trend toward worse results with increasing storage time, we do not yet know the upper limit of storage time attainable with the IP technique.**

Preliminary study of HEP levels in a human heart.

We were also able to study the effect of IP on one human heart. The heart was cardioplegically arrested and stored at 0°C. A 20-min IP was performed at 4 hours of cold storage. Ventricular biopsies were taken at various time points for HEP determination by ^{31}P-MRS. **Figure 8** shows ^{31}P-MR spectra of left ventricular biopsies taken just before and after IP. There was hardly any detectable PCr and very low levels of ATP in

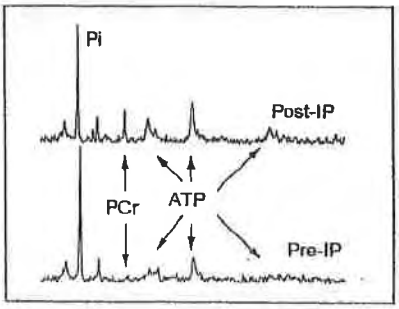


Figure 8: Human heart HEP after IP.

the pre-IP spectrum. In contrast, the post-IP spectrum showed significant elevation in both PCr and ATP and attenuation of the Pi peak. This observation gives evidence that IP can recharge HEP even in old-stored human hearts

The sum of the evidence summarized above, derived using a rigorous large animal model and in one case an actual human heart, convinced us of the value of attempting to develop IP as a clinical modality for the preservation of human hearts. We therefore proposed in Phase 1 to develop an automated IP device that would bring us closer to practical implementation of this concept in clinical medicine. The following is a progress report on the results of that effort.

Phase One Progress Report

Phase One project period. The initial funding date of Phase I of this research was August 15, 2002. The original ending date was July 31st, 2003, but a requested extension of the project to July 31st, 2004 was granted.

Key personnel, titles, dates of service, and number of hours devoted to the project.

Gregory M. Fahy, Ph.D., Principle Investigator; 1/2003-12/2003, 10% effort

Brian Wowk, Ph.D., Project Engineer, 1/2003-12/2003, 15% effort

Gary Carpenter, Project Machinist, 1/2003-5/2003, 50% effort

Tingchung Wang, Ph.D., Principle Collaborating Investigator, 4/2003-12/2003, 40% effort

Todd Massey, M.D. Transplant Surgeon, 4/2003-12/2003, 4% effort.

Summary and significance of work completed.

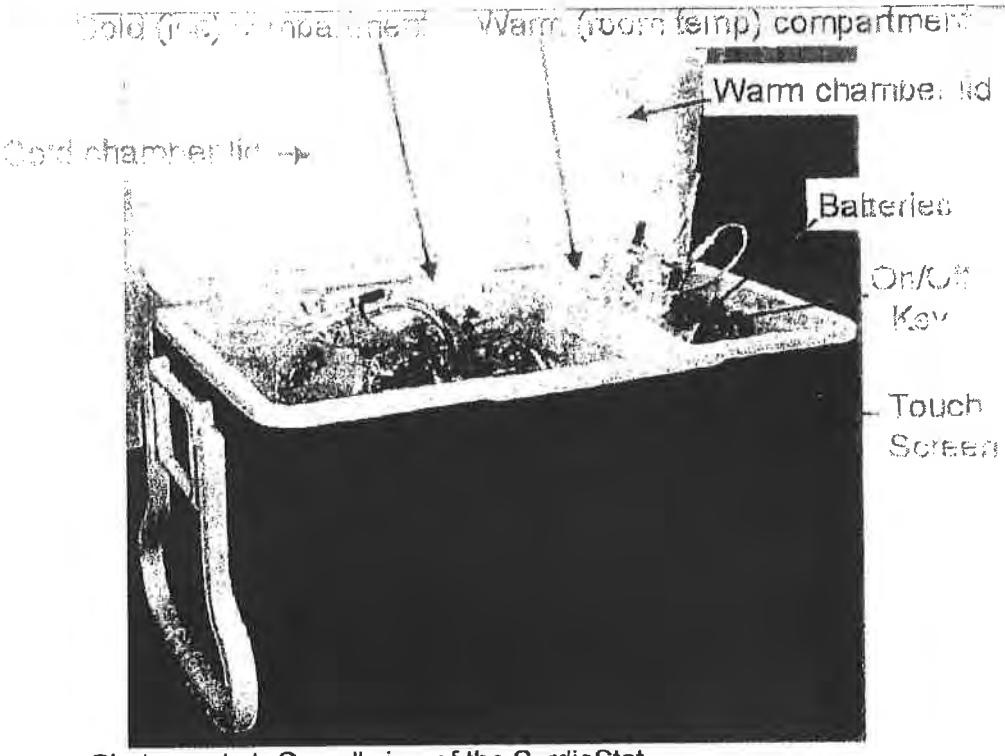
The aims of Phase I were to construct a prototype automated device capable of inducing controlled intermittent perfusion of canine or human hearts **(Aim 1)** and to test the device on canine hearts **(Aim 2).** Aim 2 was specifically to determine whether a) the device would perform as designed, b) hearts preserved with the use of the device would perform as well as hearts preserved without the use of the device, and c) the device was practical for research and medical personnel to use.

Specific aim 1 – Construction of an automated IP device

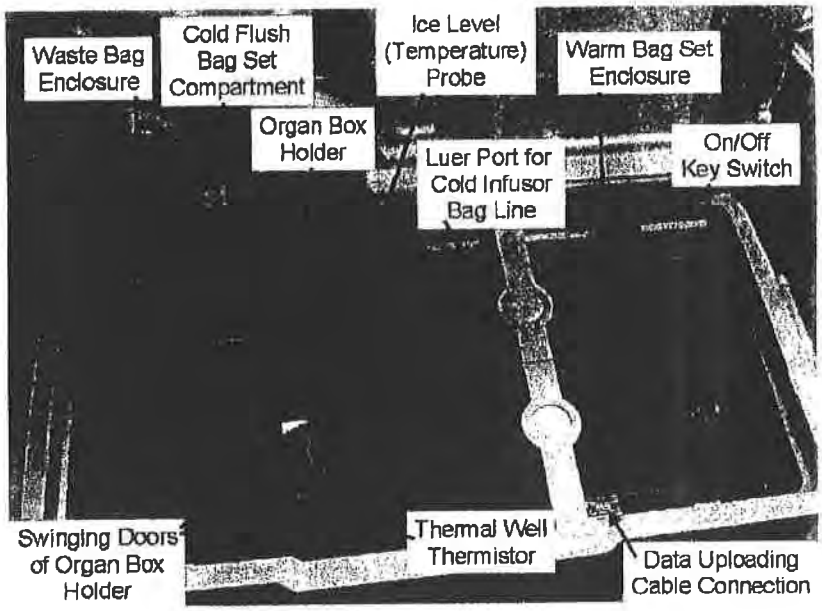
The automated IP device designed and constructed at 21st Century Medicine (the CardioStat) followed the basic design described in the Phase I application, with some improvements made possible by additional experience. Only a general overview of the construction of this device can be provided here.

The CardioStat was designed to deliver one 4 liter flush of warm (room temperature) perfusate at a pre-programmed time, immediately followed by a 2 liter flush of cold (0°C) perfusate to bring the heart temperature back to appropriate hypothermic storage temperatures. The device controls perfusion onset, duration, temperature, and pressure. The temperature, pressure, and time data are continuously logged and available for later transfer to a computer if desired. Consistent with our originally-submitted design, perfusion is driven by small air pumps that inflate air (infusion) bags that in turn compress perfusate bags to deliver perfusate at a controlled pressure. Pneumatic infusion makes the system very safe, energy efficient, and light-weight compared to peristaltic pump designs and intrinsically guards against overpressurization. The CardioStat is capable of preserving canine and human hearts for 24-48 hours without external power.

Photograph 1 shows the overall appearance of the CardioStat. The CardioStat contains two chambers, one for storing the heart surrounded by ice on the left, and one for storing the "warm" perfusate at room temperature on the right. The touch-sensitive control/monitoring screen can be seen on the right outer wall of the CardioStat. The CardioStat comes with a tote handle and wheels to facilitate transport. Its

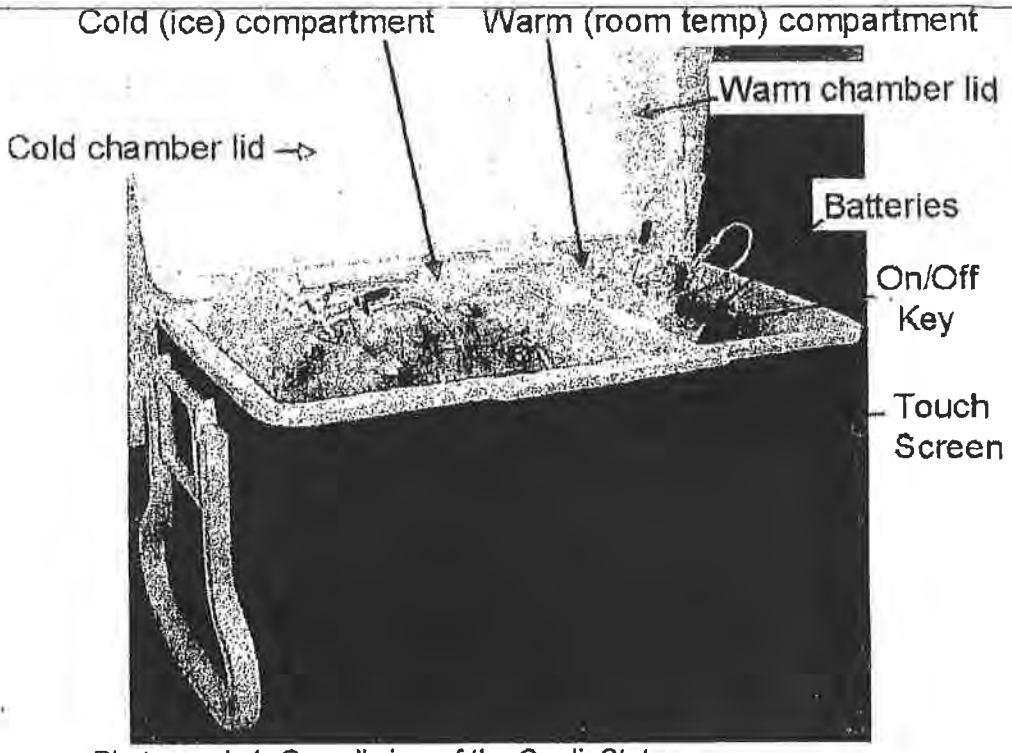


Photograph 1: Overall view of the CardioStat.



Photograph 2: Inside view of the CardioStat with no bag sets and no organ container.

insulation is sufficient to maintain ice above the organ container on the right side of the device for more than 24 hours. The CardioStat utilizes three disposable fluid bag sets (visible in Photograph 1), one for delivering warm perfusate, one for delivering cold perfusate, and one for collecting venous effluent (waste). The waste bag consists of only one large bag and an appropriate inlet, whereas the perfusate bags are bonded to the infusion bags to form bag sets. All bags are designed to easily drop into the CardioStat at the beginning of



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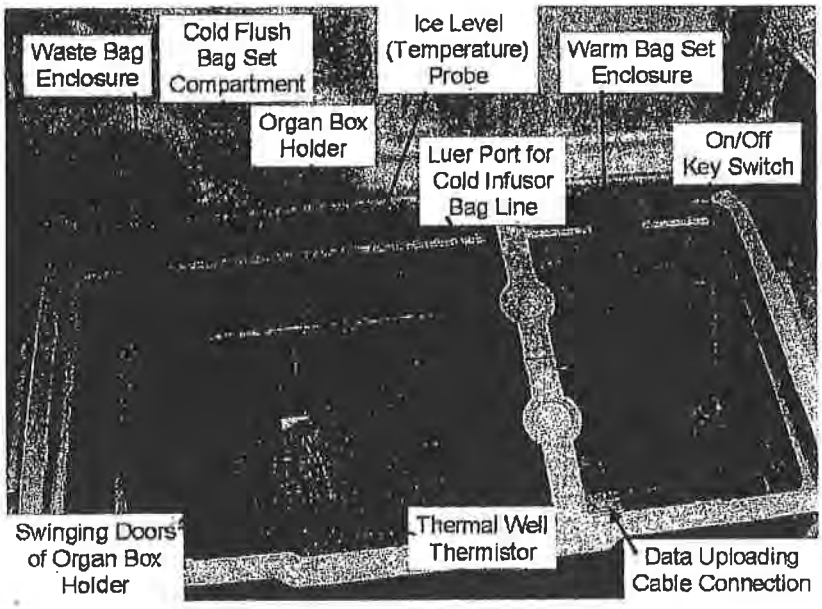

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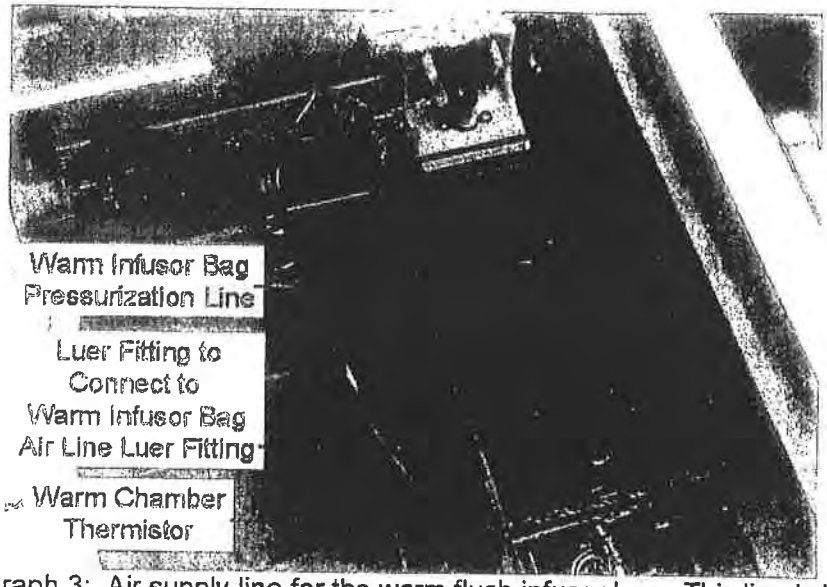
cardiac preservation and to be easily removed and discarded at the end of each preservation. The plastic compartments that hold these bags are shown in Photograph 2. Photograph 3 provides an impression of the air delivery system that drives perfusion, and Photograph 4 shows the setup window of the SmarTouch ontroller board touchscreen, with "setup," "prime," "run," "upload," and "sleep" buttons allowing different key functions to be carried out, and with control buttons for setting up the flush time and flush pressure. The controller has no moving parts.



Photograph 3: Air supply line for the warm flush infusor bag. This line is lifted to admit the warm bag set and then connected to a Luer fitting on the top of the infusor bag using the connector shown.

Photograph 4: Opening touch screen display.

The operating program is stored in non-volatile FLASH memory, and data is stored in battery-backed RAM that retains data when power is turned off. This permits the device to be turned off and on at any time, even in the middle of a perfusion cycle, with no loss of data or control disorientation. The CardioStat can be powered by batteries or by a 12V AC adapter to conserve battery power, but is designed to operate under field conditions with no external electrical power for a complete preservation cycle when this is needed.

The rigid, sterile organ box, together with the fluid bags and their connecting lines, constitute a sealed nvironment that is isolated from the rest of the device and is ultimately disposable. The organ container ermetically seals via a rubber gasket. As perfusate drains from the heart, it compresses the air within the

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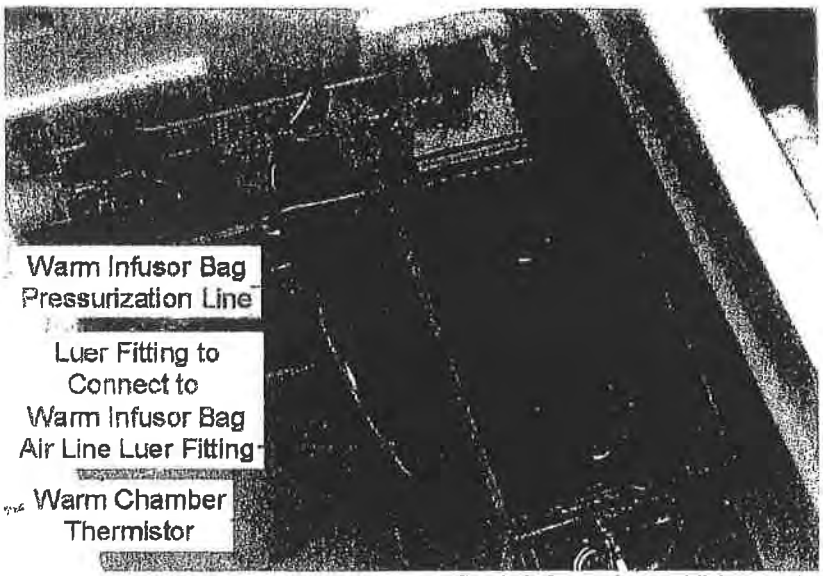

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organ container, thereby raising pressure within the box. This interior pressure (approximately 20 mmHg) drives waste perfusate up and out of the waste drainage pipe to the waste bag, leaving the heart sitting in only a shallow puddle. This pressure does not act as a venous pressure because it is uniform and hydrostatic around the heart. The heart experiences zero venous pressure, as if it were draining out into open air. The interior box pressure does, however, subtract from the applied perfusion pressure to generate a lower net effective perfusion pressure.

This device was constructed and sent together with complete instructions to Dr. Wang at the University of Rochester for evaluation and testing in Specific Aim 2.

Specific aim 2 – Testing the IP device

Sub-aim 1: Device Performance

The first question asked in Specific Aim 2 was, did the device function as it was designed to? Donor dog hearts were arrested with UR-Flush solution and transferred to the IP device. The device was programmed to deliver a single 30-min room temperature bout of IP at 10 hours of storage and then to return cardiac temperature to below 4°C for the balance of the 24-hour total storage time. It accomplished these functions and all intended data recording and downloading functions successfully in each case. The following representative figures describe the typical performance of the device in delivering first a warm and then a cold bout of IP according to the preset program.

Figure PR-1A shows the infusion bag pressure data recorded by the IP device during the entire device storage time of a dog heart. The heart was attached to the device 2 h after it was recovered from the donor dog. The line pressure of both the warm and cold infusor bags were practically at zero for the entire course of storage, except during IP. As the warm perfusion started at 10 h of storage, the pressure of the infusor bag for the warm solution rose immediately to the set pressure of 90-100 mm Hg and remained at the set pressure for the programmed 30 min of warm perfusion. After warm IP, pressure in the warm solution infusion bag rapidly returned to zero and cold perfusion immediately began, as registered by a rapid rise in cold infusion bag pressure. Cold perfusion, which was necessary to return cardiac temperature to below 4°C, lasted 15 min as programmed. Infusion bag pressure is an indirect measure of perfusion pressure that is noninvasive and requires no complicated set-up but may require correction for pressure drops in the perfusion path that could only be measured during actual testing of the prototype device.

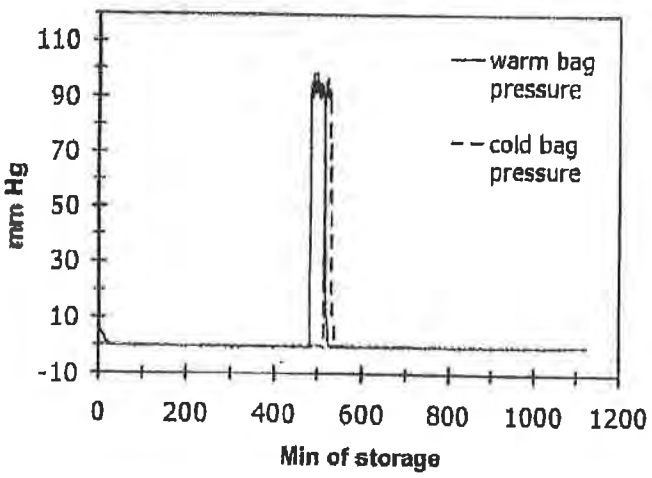
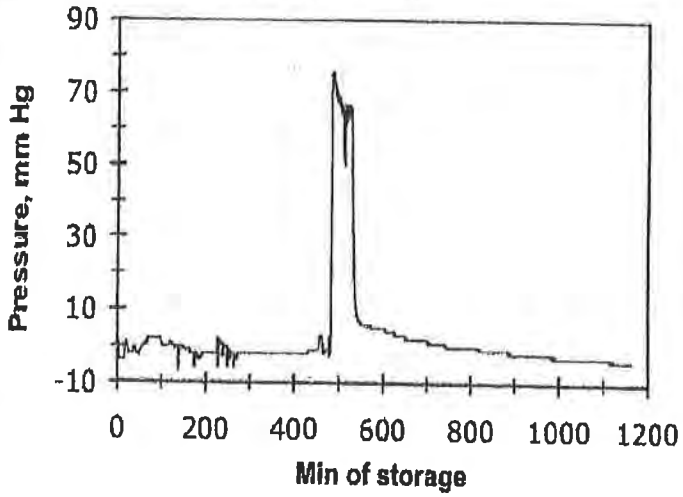


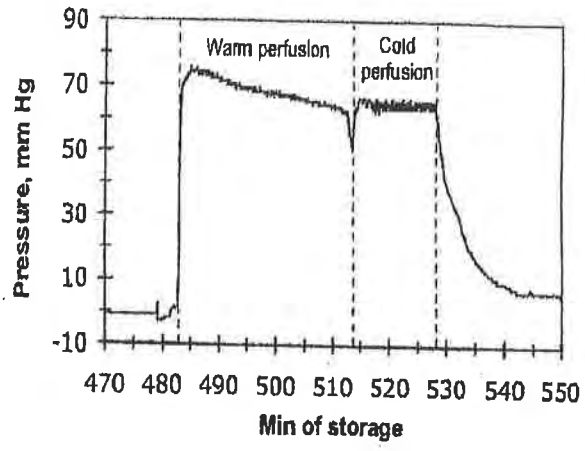
Figure PR1. A: Infusion bag pressure.



B: Aortic pressure

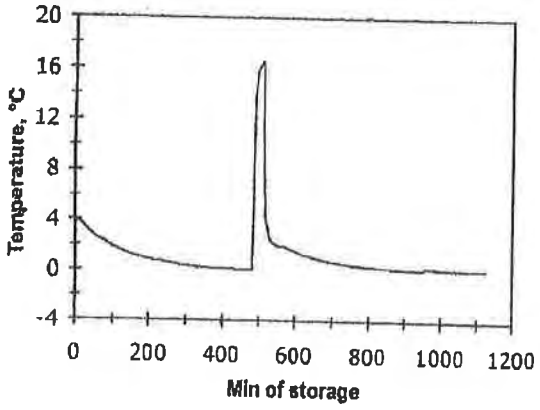
Figure PR-1B shows the pressure as recorded from a traducer located on a side arm of the aortic cannula assembly. During the first 8 hr in the machine (i.e., the last 8 hours of the total 10-hour pre-IP period), the pressure was at essentially zero. Pressure rose quickly when warm perfusion started. It briefly reached 75 mm Hg and then settled down to the desired steady value of 60-70 mmHg as the warm bag slowly emptied. After warm perfusion ended, the line pressure dropped momentarily as cold perfusion started. Aortic pressure then went up during cold perfusion to the desired 65 mm Hg and remained constant for the entire period of cold perfusion. These changes in aortic pressure during warm and cold IP bouts are shown more clearly in **Figure PR-1C**.

Comparing Figures PR-1A and PR-1B indicates that the perfusion pressure at the aorta closely tracks the infusion bag pressure during each of the two IP phases, with an appropriate offset for the pressure drop along the flow path. Therefore, infusion bag pressure can be used as an effective surrogate measure of the actual aortic perfusion pressure during both warm and cold perfusion bouts. This potentially allows avoidance of expensive disposable pressure sensors in the fluid path that require manual attachment to the electrical monitoring system. Further, the difference between absolute infusion bag pressure and absolute aortic pressure could presumably be reduced by using larger tubing conduits between the two sites.



C: Detail of aortic pressure changes.

A similar comparison is shown in **Figure PR-2**, which depicts the relationship between temperatures recorded at the floor of the heart chamber, where thermal events are induced by changes in the temperature of the cardiac effluent, and temperatures in the pulmonary artery, which are less influenced by contact with



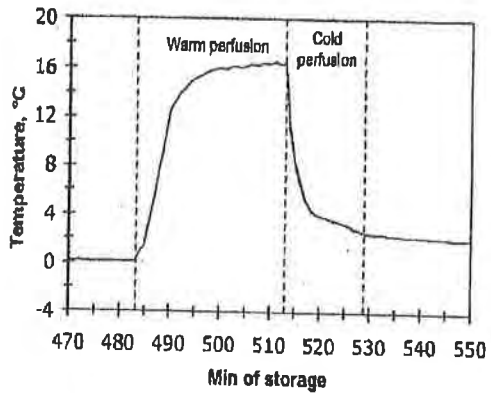
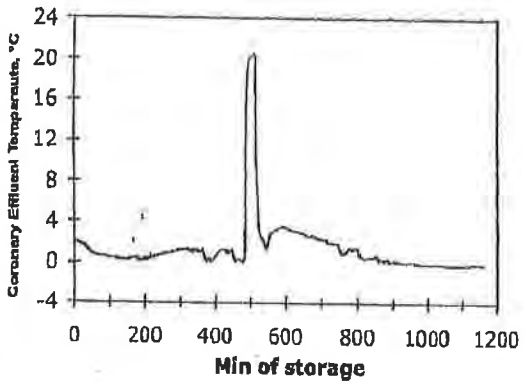


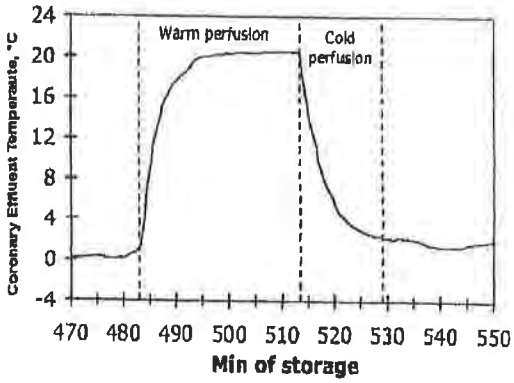
Figure PR-2. A: 24-hr heart chamber temperatures. B: Detail of chamber temperatures during IP.

the 0°C environment and previous effluent. Figures **PR-2 A and B** show the temperature of the perfusate resting on the floor of the heart chamber during cold storage and perfusion. Chamber floor temperature was allowed to exceed 0°C at zero time, but gradually declined to zero during the first 8 hours of machine cold storage. Temperature rose quickly to 16.5°C during warm perfusion, reflecting the entry of warm solution into

the heart. Temperature at the floor did not reach 25°C due to heat loss to the heart itself, heat loss to the ice-cooled floor of the chamber, and dilution by a thin layer of cold perfusate remaining on the floor of the chamber, but was an unequivocal marker of the event of warm IP. Cold perfusion rapidly returned floor temperature to a safe value (<4°C) for subsequent storage, temperature then gradually declining to zero over about 8 h after cold perfusion. Figures **PR-2 C and D** show temperatures recorded in the pulmonary artery as a marker of coronary effluent temperature. During the first 8 hours of machine cold storage, the temperature was between 0 and 2°C. When warm perfusion started, the temperature rose rapidly to 20.5°C and remained at that temperature for the entire warm perfusion period. Cold perfusion brought the temperature down quickly to 2°C. After cold perfusion ended, the temperature rebounded but did not exceed a safe value of 4°C and thereafter slowly declined to 0°C. The post-perfusion rebound could presumably be prevented by a slight extension of the cold flush period (e.g., to 20 min vs. the 15 min used in this study) if future research should indicate this is desirable.



C: 24-hr coronary effluent temperatures.



D: Coronary effluent temperatures during IP.

Sub-aim 2: Quality of Preservation Achieved Using the IP Device

After a total of 24 hours of storage (10 hrs contact with UR-Flush before IP, 30 min of warm IP, 15 min of cold IP, and 13.35 hours of subsequent storage), CardioStat-preserved hearts (n=4) were transplanted orthotopically into a recipient animal and hemodynamic performance of the recipient dog was monitored over a period of 6 hours after weaning from cardiopulmonary bypass. Control hearts (n=4) were arrested with Celsior cold storage solution (Sangstat, Inc. Fremont Ca), stored on ice for 4 hours, and transplanted and evaluated in the same way. The following table summarizes the results (values shown are means and standard errors). There were no significant differences between the results for the two groups, and the data were generally particularly close six hours after transplantation. This suggests that the hearts preserved by the IP device for 24 hours had the same functional capacity as those stored for 4 hours using Celsior solution despite the pilot nature of these experiments. As this report is being written, one more heart remains to be added to each group, and this will be accomplished before the end of the extended Phase 1 project period. We also verified that the IP device did not cause any damage to the hearts relative to the effects of storage in URS without IP by comparing the results of 24 hour URS storage with and without IP. The systolic and diastolic pressures at the end of 6 hours were identical with and without IP (Table PR-1).

Table PR-1 Hemodynamic function of hearts after preservation with and without machine IP.

Group	Hours	Heart rate	SP	DP	Max dp/dt	Min dp/dt
Celsior (4 hr)	1	104±6	99±8	59±2	946±39	-446±41
	2	99±5	125±19	87±23	973±124	-446±12
	3	101±7	1122±11	74±15	1151±142	-392±12
	4	89±3	120±4	75±15	1110±108	-459±64
	5	99±7	118±7	78±12	1066±143	-462±67
	6	96±7	116±9	72±6	1060±114	-478±22
CardioStat (24-hr)	1	102±3	115±5	77±6	899±141	-419±87
	2	101±1	111±6	68±4	963±151	-474±79
	3	108±3	93±10	56±4	796±67	-455±107
	4	104±11	97±6	58±1	888±113	-518±80
	5	108±3	97±8	56±4	1001±102	-576±44
	6	108±4	113±5	66±4	1076±107	-582±34
URS (24-hr)	6	N/A	112±13	67±8	N/A	N /A

(N/A: not measured)

Sub-aim 3:. IP Device Ease-of-Use and Design Evaluation

Our third sub-aim was to evaluate ease-of-use issues and any other aspects of device design that could be improved. The following results were obtained.

Ease-of-use experience:

- It took about 5 min to prime the perfusate bags after oxygenation and 5 min to prime the perfusate lines. By changing the bag design, these two procedures should become synonymous, and by modifying the perfusate line fittings and tubing, faster debubbling should be possible.
- Cannulating the heart took ≤ 3 min, which was not considered a problem.
- The time required to install the heart chamber in the ice compartment, which was designed to be approximately zero min, was increased due to procedure modifications at the University of Rochester (UR) that involved placing the chamber deeper in the ice, resulting in a 5-10 min placement process. New design changes should again reduce the chamber placement time to virtually zero min while fulfilling the desire of UR personnel to bury the chamber deeper.
- The time needed to retrieve the heart from the heart chamber was only 2 min or less and was not an issue.
- The time needed to clean the device after use was about 30 min. While not a particular problem, the design changes contemplated for increasing the ease of chamber installation should also greatly shorten the needed device clean-up time.

Other design issues:

- It was initially intended that hearts be perfused using a cardioplegia needle inserted into the aorta on a flexible attached line to mimic the method by which cardioplegia is induced clinically. However, the UR group ultimately preferred to have a rigidly fixed cannula entering the wall of the organ chamber onto which the aorta could be placed and ligated. This design change was made successfully, and venting issues derived from this design change were successfully avoided.

- The UR group was uncomfortable with the non-invasive measurement of effluent temperature at the floor of the heart chamber as the sole indication of cardiac temperature. This measurement was made using a non-disposable thermistor positioned to fit into a thermal well built into the floor of the organ chamber when the organ chamber was inserted into a positioning box in the device. For this reason, a fine thermocouple was also used to directly monitor and record coronary effluent temperature. As shown above, this successfully verified that the effluent temperature was appropriate, and allowed us to determine the relationship between floor temperature and coronary effluent temperature. In the future, a thermal well can be installed directly in the rigid arterial cannula, or a disposable thermistor can be built into the cannula if the extra cost and the extra step of connecting the disposable thermistor to a monitoring jack are not prohibitive.
- Our experience allowed us to relate infusion bag pressure to the actual perfusion pressure of the heart, but some discrepancy in calibration was noticed during the early stages of warm perfusion. We believe better coupling of the infusion bag to the perfusate bag and recalibration of the pressure transducers should reduce or eliminate this discrepancy. In any case, the discrepancy is not evidently physiologically detrimental. If desired the initial warm infusion pressure could be lowered or a low initial warm infusion bag pressure could be elevated linearly with time to offset the linear decrease in actual perfusion pressure. Although we prefer indirect pressure measurement to direct aortic line pressure measurement for the same reason we prefer indirect temperature measurement to direct intra-aortic temperature measurement, direct measurement is possible if the transplantation community prefers it.
- Our prototype was built using a commercially available ice chest. It would be desirable to modify the ice chest to incorporate two more wheels and a handle that would allow the ice chest to be pushed rather than pulled.
- Although the device is designed never to require cleaning of the perfusate bag compartments, there is always the chance that someone will spill fluids in these compartments that must be cleaned, and originally these compartments could not be removed for cleaning. We have therefore developed a new design concept that will eliminate all cleaning issues in the next generation device.

Significance

Our Phase 1 results provided a proof-of-concept for the feasibility of developing an automated IP device by clearly demonstrating that an automated IP device can preserve canine hearts satisfactorily for at least 24 hours. Our total body of experience implies that, in fact, these hearts would have remained viable and functional even after 34 hours of storage (24 hours beyond IP). However, we chose to limit the IP onset time to 10 hours and the total preservation time to 24 hours in order to be consistent with our marketing plans, which do not call for claims of preservation in excess of 24 hours for initial clinical introduction. The Phase 1 experiments also successfully identified certain design changes which will be able to improve both ease of use and device weight, performance, and attractiveness, as intended. This experience will be valuable to us in moving forward into Phase II.

It is also apparent from Table PR-1 that University of Rochester solution (URS) can preserve hearts as well as the combination of URS and IP as long as preservation is limited to 24 hours. This implies that an IP device is needed only for preservation beyond 24 hours. While we believe 24 hours is an inadequate preservation window for non-emergency cardiac transplantation, it is dramatically superior to the current frenetic 4-6 hour clinical limit. Therefore, delivering 24-hour storage to the transplantation community without the extra costs and education involved in using an IP device would be an attractive initial step. For this reason, we plan in Phase 2 to demonstrate the utility of URS alone for 24-hour storage using post-transplant survival as an end point, and we plan to investigate the limits of utility of URS alone for the preservation of human hearts. However, continued research on human hearts may very well show that, unlike the hearts of healthy canine subjects collected under ideal conditions, human hearts available for research and arrested using relatively ineffective solutions such as UW solution or Celsior may require IP

in order to be preserved successfully for 24 hours. We will continue to develop IP as an extremely powerful preservation modality in Phase II for this reason and as a second-generation product that may ultimately extend the attainable clinical preservation time from 24 hours to 48 hours or beyond.

No manuscript based on these Phase 1 results has yet been submitted for publication, although we will certainly publish our results when the last two planned heart transplants have been completed.

D. RESEARCH DESIGN AND METHODS

Specific Aim I: Demonstrate 3-month survival of recipients of hearts preserved for 24 hours with URS without IP.

Rationale. Excellent function of dog hearts preserved for 24 hours with URS has been demonstrated in acute transplant experiments by the UR group, but there is no proof that a heart so preserved will support life chronically. Before human clinical trials of URS can be undertaken, it is imperative to demonstrate life support function for a significant period of time postoperatively.

Experimental protocol. In human cardiac transplantation, the highest mortality occurs within the first month after surgery. Acute cardiac pump dysfunction from graft failure and acute organ rejection are major causes of death clinically (59, 60). Therefore, a 3-month postoperative follow-up period should be sufficient to indicate the acute risks of cardiac transplantation after preservation with URS. Ten transplants will be done in this group. This number is needed to obtain a reasonable feeling for the success rate of the procedure. If 10 out of 10 transplants are successful and all hearts function well, we will know that the failure rate is likely to be much less than 10% and we will have evidence for the reproducibility of positive functional outcomes in a series of convincing size.

Canine hearts will be flushed with URS, stored for 24 hours, and then transplanted orthotopically. After reestablishment of blood flow, rhythmic heart contraction, and adequate blood pressure, the recipient will be weaned off cardiopulmonary bypass, the chest will be closed, and the recipient will be nursed to full recovery. Post-operative follow-up will be as described in section F, Vertebrate Animals. After 90 days of follow-up, the chest will be opened with the dog on a respirator, the animal will be euthanized by cardioplegic cardiac arrest, and biopsies will be immediately taken from the right ventricle, the left ventricle, and the septum for metabolite and histological studies.

All of the studies to be carried out in Aim 1 will be conducted at the University of Rochester, where the animal protocols are already in place and approved and the facilities and staff are available.

Specific Aim 2: Development of an in vitro testing system for human hearts, and testing of the system using monkey hearts

Rationale. Although canine hearts are very well preserved after 24 hours of simple cold storage with URS and are highly likely to support life, there could be species differences between dogs and humans that would make preservation of human hearts less effective. It is therefore highly desirable to develop evidence that the benefits of URS also apply to human hearts. While it is not possible to prove that human hearts can support life prior to conducting human clinical trials, it is possible to show that human and monkey hearts can develop adequate function in vitro. Consequently, it is the goal of Aim 2 to create an in vitro cardiac evaluation system for human hearts and to validate it using primate hearts prior to obtaining actual human hearts for study. In addition to allowing us to show viability of a primate heart after preservation with URS, the use of vervet hearts in these experiments will provide us with a supply of hearts for evaluation that will be of predictable quality and will be available at predictable times, unlike the human hearts to be studied in Aim 3.

Establishing the normothermic perfusion and in vitro testing device and methods. 21st Century Medicine (21CM) will consult with Dr. Wang in order to obtain his assistance in establishing a working blood perfusion machine for human hearts with full cardiac evaluation capability and to train our staff in the use of this device and in the measurement of cardiac performance in vitro. Dr. Wang will make at least one trip to 21CM during the development of the device and techniques, and will assist us in all aspects of establishing testing equipment and methodology at 21CM. Dr. Wowk will create the computer interfacing required for device operation and data capture and Mr. Carpenter will fabricate custom parts as the need arises and assemble parts. Dr. Wang will guide design details for the device in collaboration with Dr. Fahy and will advise us concerning the acquisition of necessary components and testing device subsystems, and will help us prepare written instructions for operating the device and for measuring cardiac function. Dr. Wang will also conduct the first two monkey heart experiments at 21CM as both a debugging exercise and as a training experience for other 21CM staff, and will help us establish manual IP methods at 21CM. He will also play a major role in writing up all results associated with both Phase 1 and Phase 2 results.

The computer-controlled and monitored "blood" perfusion system at 21CM will be based on a manual system presently in existence in Dr. Wang's laboratory at UR, on literature descriptions of successful blood perfusion devices for hearts (55, 66), and on our own experience in designing and constructing a blood perfusion machine for the evaluation of kidneys (68-71). The heart, reservoirs, and pumps will be housed in an incubator at 37°C. The arterial perfusate will be saturated with oxygen, and aortic and pulmonary artery oxygen sensors will permit oxygen consumption to be calculated from the transcardiac pO2 gradient, the coronary flow rate, the initial weight of the heart, the hematocrit, and the human oxyhemoglobin dissociation curve as previously described (68) and displayed in real time. The system will include a venous reservoir coupled to a computer-controlled pump able to deliver perfusate to the left atrium at a computer-predefined pressure so as to establish desired preloads, with continuous computer documentation of atrial perfusion rate derived from roller pump speeds. (The PI has found, in the kidney blood perfusion system, that roller pumps will not damage perfusate erythrocytes over the period of observation planned in these studies (69) Atrial perfusion rate in this case will be equal to cardiac output. A TransSonic electromagnetic flow probe will continuously quantitate the flow rate of perfusate from the pulmonary artery, which will be equal to the coronary flow rate. Additional recording instrumentation will monitor perfusate pH, pCO2, and left ventricular pressures (see Detailed Procedures, below). Oxygen will be added and produced CO2 will be removed using a clinical membrane oxygenator which will be reused once before being discarded. The circuit will include an in-line blood filter (4C7730, Fenwal).

Other end points. Perfusate samples taken every 15 min will follow enzyme leakage, perfusate electrolyte (calcium, phosphate, magnesium, sodium, and potassium) levels, perfusate substrates, and lactate levels. At the end of the perfusion, the heart will be weighed and biopsies will be taken for water content and HEP determination (see below) and the heart will be perfused with low-osmolality Karnovsky's fixative for light and electron microscopic follow-up.

Perfusate. The perfusate volume will be approximately 15 times the volume of cardiac tissue to avoid the exhaustion of perfusate substrates and the buildup of any metabolic byproducts. Based on studies of the depletion of perfusate substrates (glucose and acetate), additional continuous substrate addition protocols will be established should this prove necessary. For a 100 gram heart, the perfusate volume will be about 1.5 liters, consisting of 1 liter of washed erythroctes in normal saline (at a hematocrit of 45%) and 0.5 liters of an additive solution that will reduce hematocrit to 25-30%, supply substrates, establish pH buffering, and provide an appropriate electrolyte (including physiological calcium) and colloid balance (see Detailed Procedures section). White cells will be removed from the erythrocyte perfusate using a Sepacell R-500 leukocyte filter (Fenwal) shortly before use.

Primate Products ?

System evaluation using vervet hearts. Veterinary staff at the Behavioural Sciences Foundation (BSF) monkey colony in St. Kitts will be taught the technique of in situ cardiac perfusion by Dr. Fahy. Dr.

Fahy will perform the first procedure with the help of BSF staff, and BSF staff will perform the second procedure with the help of Dr. Fahy on the same day. These hearts will be returned to 21st Century Medicine by Federal Express or Sterling Courier on the day of procurement for arrival and testing the following day at 21CM by Dr. Wang. Subsequent procedures will be performed entirely by BSF staff and similarly sent to 21CM for testing the following day. We assume the first two hearts will be needed for debugging the perfusion device and our perfusion technique, and that the remaining 5 hearts will provide valid physiological performance data demonstrating the successful preservation of functionality. The details of the procedure for obtaining hearts flushed with URS are given in section F., Vertebrate Animals. We are currently in touch with BSF by email and telephone, and can therefore plan and coordinate the procurement and the testing procedures in advance. In these experiments, vervet blood will be collected in St. Kitts from the heart donors in standard blood bank ACD and flown to California. Upon arrival, the blood will be washed with normal saline and prepared at a hematocrit of 45% prior to being diluted by our blood diluent. We have ample large centrifuge capability to permit red cells to be sedimented and separated from the buffy coat at 600 g for 10 min for resuspension in normal saline according to standard methods.

Specific Aim 3: Human heart evaluation in vitro after 24- hour storage

Rationale. The combination of life support by 24-hour preserved dog hearts, functional recovery of 24-hour preserved, long-distance transported monkey hearts, and functional recovery of 24-hour preserved human hearts should provide virtually the best possible evidence for the appropriateness of human clinical trials of URS. The proposed studies of Aim 3 would also demonstrate that URS is practical to employ within the existing clinical system of heart allocation even given present handicaps resulting from the current lack of use of URS for cardioplegia prior to organ procurement. Finally, the proposed studies will provide direct comparisons between URS, UW solution, and Celsior, which will be important for proving equivalence or superiority to these predicate devices for purposes of eventual FDA approval.

Cardiac procurement. Because of the importance of direct evaluation of human hearts, including comparisons between hearts preserved with URS and with competing cold storage solutions, and because of the difficulty of obtaining human hearts at the University of Rochester (UR), it is necessary to expand our studies to include hearts obtained from sources other than UR. We have made arrangements to obtain human hearts from two leading organ procurement services in addition to the Finger Lakes Organ Recovery team at UR, and will go to other sources if necessary to obtain additional human hearts for these studies. Most hearts not originated at UR will be studied at 21CM.

UR will provide at least five human hearts for the proposed research. At UR, Dr. Todd Massey, who is a human heart transplant surgeon, and the Finger Lakes Organ Recovery team will arrest available human hearts using URS and provide them to 21CM via Sterling Courier when timing makes this feasible. When a heart is available and it is impossible for it to be sent to 21CM for arrival within 24 hours of collection, it will be evaluated by blood perfusion in Dr. Wang's laboratory at UR. By studying the condition of human hearts preserved with URS for 24 hours at both UR and at 21CM, we can in theory control for the effect of any possible damage sustained as a result of shipping. Unfortunately, hearts available from UR will be available only rarely, and if only 5 are available per year, we will have to pool the UR results and the 21CM results and leave the issue of transport injury unresolved.

One major source of hearts other than UR will be the National Disease Research Interchange (NDRI), a nationwide service for supplying human cells, tissues, and organs for scientific investigation. NDRI works closely with existing organ procurement organizations (OPOs) to identify organs that meet the criteria of investigator research protocols. Upon learning of a candidate organ, the investigator is telephoned by NDRI, told of the particular circumstances of that specific organ, and asked if the candidate organ is wanted. If the investigator accepts the organ described by NDRI, NDRI notifies the OPO to collect it. Generally, the OPO does this using cardioplegia with UW solution. Although this is not ideal for our purposes, we can still use ...ch hearts as controls, and we can also reflush them with different solutions to allow these different ...olutions to be compared to UW and to each other (see below). Further, NDRI will investigate the possibility

of inducing cardioplegia with URS or Celsior instead of with UW solution, or of back table reflushing with these solutions. Once the heart is flushed, it is packaged for ice transport. After notifying the OPO of the decision to accept a specific heart and advising the OPO of the flushing technique to be used, NDRI then notifies Sterling Courier, a service we have used in the past, to deliver the organ by either air or ground transport. We are located 15 minutes by car from the Ontario International Airport in Ontario, California, and are also accessible through local airports in Riverside, San Bernardino, and Corona, and can receive deliveries from Los Angeles International Airport by car within 1.5 hours and from John Wayne airport in Orange County within about 1-1.5 hours. We are also able to accept organs by direct ground transport from Los Angeles (One Legacy Transplant Donor Network and the Doheny Eye and Tissue Transplant Bank) or from San Diego (Life Sharing), which is advantageous because ground transportation can be arranged at any time of the day or night, whereas air transport depends on the availability of commercial flights within a narrow time window near the moment of collection of the heart. Although we will accept organs from any feasible source, we expect that the majority of our hearts will arrive by ground transport. In most cases, hearts are available for transport anywhere from 45 min to 2 hours after initial cardioplegia, and transport times from either Los Angeles or San Diego to 21CM by courier will require less than 2 hours, so it should be feasible to obtain most hearts from NDRI sources in 4 hours or less. Hearts will be accepted if they meet our criteria (see Detailed Procedures, subsection 4, below) and can arrive at 21CM within 4 hours of cardioplegic arrest with UW solution. If our results are good at 4 hours and we find that it is necessary to expand our time limit in order to obtain sufficient hearts, we will extend our cutoff time to 5 and then to a maximum of 6 hours. If hearts clearly behave abnormally after being obtained after greater than 5 hours of cold cardioplegia in UW solution, these hearts will be categorized separately.

Our third source of hearts will be the International Institute for the Advancement of Medicine (Jessup, PA), or IIAM. IIAM also works with OPOs in our area of California as well as with OPOs in Seattle, Denver, Los Vegas, and Phoenix that can also supply us if appropriate flights are available. The terms of availability from IIAM are the same as for NDRI, but IIAM has in addition proposed moving hearts from New York, New Jersey, Maryland, and Pennsylvania into its laboratory in New Jersey and treating the hearts there as we would treat the heart in our laboratory at 21CM (see below). This would then theoretically increase the "shelf life" of the heart to 24 hours, which would enable the heart to be shipped to 21CM or to UR for in vitro evaluation, thereby greatly increasing the number of available organs for our study.

If hearts available through NDRI, IIAM, and UR are insufficient, there are other sources, including International Bioresearch Solutions and many others (e.g., www.resinets.com/health/transpla.htm), and we will contact as many such services as may be necessary in order to obtain sufficient hearts to complete the studies as proposed.

Preservation protocols. The hearts studied in this Aim will be allocated to one of 5 experimental groups, as follows.

URS control group. Hearts provided by UR in URS will be held at 0°C for the balance of a total 24-hour preservation period and evaluated using the methods of Specific Aim 2. The sum of hearts evaluated under these conditions at both UR and 21CM is expected to be 5. However, if NDRI, IIAM, or another service can successfully obtain additional hearts for us that have been initially arrested in and stored in URS, we will increase the number of hearts in this group to up to 10 in view of the importance of this group. The extra 5 hearts will be subtracted from the group of 5 physical model hearts of Aim 4 to keep the budget unaltered by this increase in the size of the URS control group. The goal will be to allot equal numbers of hearts to 21CM and to UR in this group to provide methodological validation by comparing results between the two laboratories.

Other hearts arriving at 21CM will be assigned to one of the following experimental groups, the nature of which will be determined by the capabilities of our organ procurement services before the beginning of our studies.

UW control group. These hearts (n=5) will be kept in UW solution at 0°C for the balance of 24 hours of preservation and evaluated by the methods of Specific Aim 2.

UW warm rescue group. These hearts (n=5) will be perfused for 30 min with oxygenated, room temperature URS at 65 mmHg using a bag of URS hung on a pole at the requisite elevation. They will then be perfused for an additional 20 min with oxygenated cold (0°C) URS at 65 mmHg using a simple roller pump to deliver perfusate from the bag buried in ice to the heart while pressure is monitored from a sidearm leading to an electronic pressure transducer interfaced to our data tracking computer. The heart will then be stored for the balance of a total period of 24 hours since original cardioplegic arrest with UW solution. Based on our experience with IP as seen in experiments such as those of Figure 6, for example, we believe IP in this case will reverse the preservation injury accumulated in the UW-flushed hearts up until the time of the IP. This should convert the stored and nearly-exhausted UW hearts into the equivalent of hearts that have been freshly arrested with URS. This should allow a second way to show that hearts preserved with URS can be preserved for 24 hours, it should provide a second way to prove that URS is superior to UW solution for cardiac preservation, and it should also demonstrate that hearts flushed with UW can be rescued by reflushing with URS. The significance of the latter demonstration will be discussed later in this section.

UW cold rescue group. This protocol (n=5) is the same as the UW warm rescue group protocol except that the first URS perfusion will be at 0°C rather than at room temperature. This protocol will test whether warm IP is necessary to resuscitate hearts after deterioration in UW solution, or whether on the other hand the simple replacement of UW solution with URS is enough in itself to stabilize the heart for the balance of 24 hours. We would not want to recommend to transplant surgeons that they use a two-step rescue procedure if one step will suffice, nor would we want to advise them to switch to cold URS without a warm perfusion step if this simpler method is inadequate.

Celsior comparison group. These hearts (n=5) will be treated in a way that will depend on our arrangements with our organ suppliers. If they are able to arrange to arrest hearts with Celsior, we will accept Celsior-arrested hearts, maintain them for 24 hours from the onset of the arrest, and evaluate them in vitro as in the earlier groups in this series. This will provide a direct comparison to the URS control group and the UW control group, enabling us to see if simple cold storage results for these three solutions differ after 24 hours of storage. If our suppliers obtain hearts that were arrested in UW but immediately switched to Celsior on a back table, we will accept these as though they were arrested initially with Celsior. If, on the other hand, our suppliers are not able to provide us with hearts that are in Celsior at the time they are brought to us, we will subject these hearts to IP with URS as in the UW warm rescue group above in order to reverse accumulated preservation injury and convert the hearts into the equivalent of fresh URS-arrested hearts. These hearts will then be flushed for 20 min with 0°C Celsior rather than with 0°C URS, after which they will be stored for the balance of a total 24-hour preservation period and evaluated as per the other hearts in Aim 3. In the latter case, we will be able to make a direct comparison between the Celsior group the UW warm rescue group. Failure of hearts in the Celsior comparison group to equal the performance of hearts in the UW warm rescue group will provide direct evidence of the inferiority of Celsior to URS. If the 30-min warm URS IP does in effect reset the clock at a new physiological time zero, then the Celsior comparison group should be the equivalent of a group that was arrested with Celsior originally and then preserved for about 18-20 hours, and can be directly compared to the UW warm rescue group, which should be equivalent to an 18-20 hour uncomplicated URS-arrested and stored group.

To recapitulate, the result of all of these experiments should be answers to the following questions. Can human hearts arrested with URS and held for 24 hours function afterwards? Are such human hearts more functional than similarly-treated human hearts arrested with and stored in UW solution? Are URS-preserved hearts more functional than Celsior-preserved hearts? Can hearts arrested with UW solution be rescued by perfusion with URS? Does the rescue of UW-preserved hearts require warm perfusion or is cold perfusion sufficient?

Even if URS cardioplegia is shown to be highly effective for the 24-hour preservation of human hearts, we must assume that its use will not be adopted by all OPOs overnight for a variety of reasons. This will presumably result in innumerable cases in which hearts can be procured with UW solution but can't be used without transportation lasting longer than the 4-6 hour "shelf life" of a UW-flushed heart. Under these conditions, many physicians will want to re-flush the heart with URS either manually or in a controlled,

automated IP device in order to salvage the heart for transplantation. For this reason, it is imperative to discover whether URS perfusion, whether at room temperature or at 0°C, is in fact able to reverse UW-nduced injury and permit extended cardiac preservation. It is also important, in this context, to know whether hearts so rescued are subsequently best stored in URS or whether it is permissible or even desirable to switch them to Celsior for the duration of ice storage.

Protocol logistics. The hearts that become available for these studies will become available at unpredictable times of the day or night and on unpredictable days of the week. Personnel involved in conducting these studies must be on call at all times, and therefore cannot be primarily responsible for other laboratory duties, and must be kept on the payroll not only during the experiments but also during the unpredictable intervals between the experiments. A new dedicated staff member will therefore be required for these studies. Sterile stock solutions can be prepared and refrigerated to enable perfusate to be made quickly by appropriate mixing of stock solutions, but stock solutions will have to be discarded at least once a week and remade, usually without having been used (solution aging experiments will be undertaken in Phase III). We assume a major expense in the replacement of outdated solutions, including the replacement of URS lots sent to OPOs for their potential but relatively unlikely use, for this reason. A separate refrigerator will be needed to store these solutions at 21CM. In addition, specific, sheltered laboratory space must be available in which to conduct these experiments. The designated space should not be used for other purposes in order to maximize protection of employees working on other projects and to ensure that the space is in a state of readiness at all times. 21CM presently lacks laboratory space of the correct characteristics for these experiments but has ample undeveloped warehouse space that can be developed, and we propose to construct a special laboratory room specifically for these experiments.

Specific Aim 4: Refinement of the IP Device

Rationale. As noted in Specific Aim 3, the use of URS IP may be required even for 24-hour human heart preservation if UW solution continues to be used as the central solution for human organ procurement. As also noted in the Background and Preliminary Results sections, IP may be able to allow human heart preservation for periods in excess of 40 hours, with potentially revolutionary consequences. Although warm IP can be induced manually using a simple hanging bag technique, the step of subsequently cooling hearts back to 0°C
using similar methods is hazardous. Medical personnel may be unaware that solution in a hanging bag will warm up considerably during the course of a 20 min cool-down period, and consequently induce some damage to the heart by storing it after inadequate cooling. Cooling the heart by using a roller pump to deliver perfusate from a bag packed in crushed ice as in the manual method of Aim 3 is very unlikely in a medical setting. In general, manual IP runs the risk of errors in timing and errors in technique. For all of these reasons, the continued development of a practical IP device remains advisable both as a backup to URS alone and as a second-generation product to eventually take the clinical market from 24 hours of preservation to 48 hours and beyond. We will improve the IP device prototype and test it on human hearts.

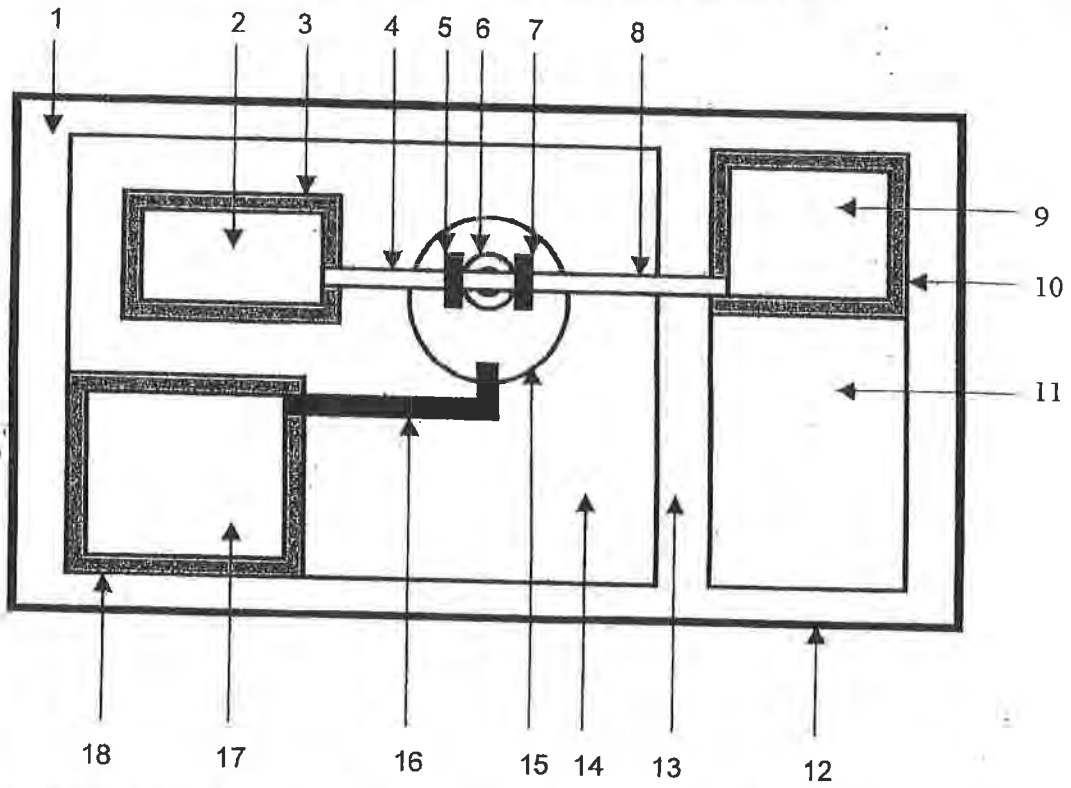
Desirable refinements in the IP device. Based on our beta testing of the IP device prototype, design changes in the IP device have been conceptualized as depicted in the following simplified schematic diagram.

Implementing the desired design changes would involve the following measures.

Modification of organ chamber and organ cannula. It is desirable to cannulate the aorta with the heart submerged in ice-cold URS to avoid the introduction of air bubbles, but it is also desirable to perfuse the heart from above on an aortic cannula rather than allowing the heart to lie on the floor of a container, where the heart's own weight could compromise superficial perfusion. We will construct a container lid that will allow the heart to be cannulated while submerged, then rotated into an upright position for perfusion without surrounding fluids. We will also construct the cannula to contain a thermal well into which a

permanent thermistor will automatically dock, thus allowing accurate recording of aortic temperature without any effort on the part of the operator.

Modification of perfusion lines. Larger-diameter tubing will be used between the perfusate bags and the heart and between the heart chamber and the waste bag, with fewer fittings, to facilitate air bubble removal, reduce dead distance, and reduce line pressure drops along the flow paths. Priming of the perfusate bags will automatically also prime the perfusate lines shortly before use.



Legend: 1 = Insulated outer wall; 2 = cold bag set in well in ice tank; 3 = cold bag set well sleeve; 4 = cold perfusate bag perfusion line; 5 = cold perfusate bag perfusion line check valve; 6 = aortic cannula, connected to organ chamber and to cold and warm perfusate bag perfusion lines; 7 = warm perfusate bag perfusion line check valve; 8 = warm perfusate bag perfusion line; 9 = warm perfusion bag set in well in warm side of CardioStat; 10 = sleeve in warm perfusion bag set well; 11 = electronics compartment of warm side of CardioStat (contains one air pump per infusion bag, batteries, microprocessor, heater, on/off switch, power leads to thermoelectric coolers, etc.); 12 = front wall of CardioStat where touchscreen display is mounted; 13 = insulated wall between warm and cold sides of CardioStat; 14 = ice/water tank (fills ice chest except for wells indicated); 15 = organ chamber, which resides in a deep well in the ice tank that is also lined by a removal sleeve for cleaning (not shown); 16 = effluent line for transferring effluent from the organ chamber 15 to the waste bag 17; 17 = waste bag residing in a well in the ice tank; 18 = sleeve of waste bag well.

Construction of thermoelectric ice tank. The device will be modified to contain a permanent sealed tank of water and air that can be converted into a solid block of ice using thermoelectric devices between preservation runs. Solid ice will occupy about 40% less volume than crushed ice and there will be no need to ever add ice to the device or to pour out water formed from melting. The organ chamber will fit into a well in the ice tank, allowing it to be located deep within the temperature controlled area without ever becoming wet. This will also prevent the chamber from moving relative to the CardioStat during transport.

Installation of removable wells for cleaning and electronics lid to avoid contamination. Waste and cold perfusate bags will fit into sleeves positioned in wells in the ice tank. Cleaning can be effected by lifting the sleeves out of the ice tank wells, cleaning them, and sliding them back into position. The warm perfusate bag set will be repositioned to be out of the way of the warm compartment electronic equipment and will slide into a similar sleeved well. The electronic components will be covered with a protective lid to prevent spills into the electronics area.

Improvements in battery support. Although it was not an issue in the Phase 1 experiments, we assume that more standard, rechargeable batteries would be more desirable than specialized single-use batteries, and that battery packs that plug in and can be taken out as units would also be more desirable.

Elimination of toting issues. We will add two casters and modify the tote handle for greater ease of use.

Testing the modified IP device. We will obtain initially 3 human hearts that are valid vascular models but not necessarily competent for physiological testing, for example hearts that have been stored in UW solution too long before reaching us to be used for physiological testing, or hearts that for other reasons are easier to obtain than hearts that meet our usual physiological performance standards. These hearts will be used as physical models to ensure proper operation of the reconstructed device with actual human hearts. Subsequent experiments will depend on human heart availability. If hearts are available, we will obtain five that have been preserved with UW solution and perform machine IP on them with URS at 4-6 hours post-arrest using a machine version of the manual IP method used in Aim 3 for the UW warm rescue group. These hearts will then be tested physiologically in the same way as the UW warm rescue group to determine whether machine IP works as well as manual IP in this protocol. If human hearts are not available, we will obtain another 5 vervet hearts according to the procedures in Aim 2. When they arrive they will have experienced several hours of cold storage in URS. We will subject them to the same IP procedure as that used in the UW warm rescue group and store them for 36 hours before physiological testing on our blood perfusion machine in order to demonstrate the ability of machine IP to produce successful storage of primate hearts for times longer than 24 hours.

Estimated Timeline of the Proposed Research

Aim 1 (survival study) will require one week of effort per transplant, and transplants will be conducted once every three weeks to accommodate Dr. Massey's schedule. Initial preparations will require another two weeks. Therefore, these studies will be completed 20 weeks (about 5 months) after the beginning of the grant period. Aim 2 (development of normothermic perfusion machine) will begin one month after the start of the grant period and should be completed 5 months later. Vervet hearts will be procured starting in month 7, and we will thereafter receive one heart per week for a total of 5 weeks. Aim 3 (testing human hearts) will begin in month 7 at UR and in month 9 at 21CM and will continue until the end of the second year. Aim 4 (refining the IP device) will begin in month 7 at 21CM, with human heart models being tested in the first quarter of the second year and with physiological testing in the 3rd and 4th quarters of the second year.

Detailed Procedures To Be Used

1. Procedures involving vertebrate animals, including heart procurement, heart transplantation, postoperative follow-up, diagnosis and treatment of rejection, assessment of cardiac function after transplantation, and euthanasia are described in **section F, Vertebrate Animals.**

2. Criteria for acquisition of human hearts - Human hearts will be accepted if they are declared unsuitable for transplantation because of ejection fraction lower than 50%, regional wall motion dysfunction, dilated cardiac hypertrophy, extensive coronary diseases, or non-deadly infectious diseases.

3. In vitro measurement of human cardiac function All human materials, including both hearts and blood, will be treated using universal precautions in a BSL-2 laboratory environment. Stored human hearts

will be perfused in vitro at 37°C with Krebs Henseleit buffer (KHB: 118 mM NaCl, 11 mM glucose, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.4 mM Na_2-EDTA, and 2.5 mM $CaCl_2$) containing human erythrocytes obtained from a local blood bank and hydroxyethyl starch (HES) for colloid osmotic support. Erythrocytes washed in 0.9% normal saline will be obtained from local blood banks. One part reconstitution solution will be added to two parts by volume of washed red cells to lower hematocrit from 45% to 30% and adjust extracellular solution composition and pH to that of KHB plus HES. A 30% hematocrit will be high enough to deliver adequate oxygen supplied at >atmospheric tension, but low enough to have a manageable viscosity in the presence of HES. Perfusate pH will be adjusted using 95% O_2/5% CO_2. HES (penta fraction, obtained from B. Braun Medical, Philadelphia, PA) will be used at a concentration of 6% w/v. The technique employed will follow previously described blood perfusion methods that have attained successful maintenance of function of isolated large animal heart for hours (55, 66). Initially, the heart will be perfused in retrograde fashion in Langendorff mode and electrically defibrillated (DC Defibrillator Model 263217, American Optical Corp) at an energy setting of 20 W sec. If multiple shock is needed, the energy level will be raised by 10 W sec incrementally up to 50 W sec. Once the heart assumes rhythmic contraction and generates aortic pressures of 70 mmHg or more, the left atrial appendage will be connected to a left atrial reservoir and perfusion will be converted to working ejecting mode. To construct a Starling curve, the preload will be set at 10, 15, 20, and 25 mm Hg and the heart will be ejecting against an afterload of 70 mm Hg and paced at 75 beats per min. The pulmonary artery will be cannulated to measure coronary flow by timed collection or flowmetry. Left ventricular (LV) pressure and heart rate will be monitored and recorded throughout perfusion using a PowerLab data acquisition system at UR or a LabView data acquisition system at 21CM. Pulmonary artery flow will be assumed equal to coronary flow and either atrial inflow rate or the sum of aortic flow and pulmonary artery flow will be assumed to equal cardiac output. Hemodynamic function including heart rate, systolic and diastolic LV pressure, ±dp/dt of LV pressure, aortic and coronary flow will be monitored and recorded continuously.

6. Statistical analysis - Data analysis by the UR group will be performed on a microcomputer using a StatView program. Between group differences will be tested by Students' one tailed or two-tailed unpaired t-test, as appropriate, unless the compared populations are not normally distributed or have unequal variances, in which case the Mann-Whitney rank sum test will be applied. Data analysis by the 21st Century Medicine group will be the same in substance, but performed using SigmaStat (SPSS, Inc., Chicago, IL). Among group differences will be tested by analysis of variance and Fisher's least significant difference test. A p value < 0.05 will be considered significant.

E. HUMAN SUBJECTS –

1. There will be no human subjects in the proposed research. All human hearts will be obtained from heart-beating human cadavers. Human hearts disqualified for transplantation but suitable for our research will be provided by the Finger Lakes Donor Recovery Network, 30 Corporate Woods - Suite 220, Rochester, New York 14623. This research is believed to be categorized as Exemption 4.
2. Donor identification is not germane to the proposed research. Therefore, donor identities will not be revealed to research personnel, thus protecting donor privacy.
3. We will keep records pertaining to the inclusion of women and minorities in the donor population, but we will have no control over the gender or race of the donors in these studies. Because of the scarcity of donor hearts, we will have to accept any heart that is available to us.
4. This is a study of adult donor heart preservation. The issue of including pediatric donor hearts is not applicable.

F. VERTEBRATE ANIMALS - The objective of this research is to better understand the effect of UR solution in preserving the functional viability of canine, monkey, and human hearts. All canine heart transplant experiments will be conducted at the University of Rochester according to their approved protocol. The facility and programs of the Vivarium an Division of Laboratory Animal Medicine of the School of Medicine

and Dentistry are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and are in compliance with state law, federal statute and NIH policy. The University of Rochester's Division of Laboratory Animal Medicine faculty consists of one board certified laboratory animal veterinarian, 2 clinical lab animal veterinarians and a veterinary pathologist. They are assisted by trained and licensed animal health technicians, as well as a staff of approximately 17 animal care technicians. The Division is responsible for providing preventive, diagnostic, and clinical services for all lab animals, providing guidance to investigators regarding handling, immobilization, anesthesia, analgesia, and euthanasia as well as monitoring of all surgery programs and the provision of appropriate postsurgical care. A veterinarian is always available during or after normal working hours, weekends and holidays. Staff are trained to recognize signs of ill health and a well-defined reporting mechanism is in place to ensure that such animals are examined by appropriate Division personnel in a timely manner. The Division's Animal Disease Diagnostic Laboratory supports the efforts of the programs that provide veterinary care. All monkey hearts will be obtained by staff at the Behavioural Sciences Foundation (BSF) in St. Kitts, BWI. BSF protocols are in full compliance with US and Canadian guidelines (see letter of support from Dr. Frank Ervin, Medical Director.)

Procurement and storage of the canine heart. Mongrel dogs (20-25 kg) of either sex will be anesthetized with sodium pentobarbital (65 mg/kg, i.v.). A median sternotomy and an anterior pericardiotomy will be performed to expose the heart while the respiration is maintained by mechanical ventilation. After heparinization (300 units/kg, iv) for 5 minutes, both the superior and inferior vena cava will be severed to vent the heart. The aorta will be cross-clamped and an infusion of 1000 ml ice-cold cardioplegic solution, URS, to the root of aorta at 80-100 mm Hg pressure will begin. After cardioplegia, the arrested heart will be excised and stored by immersion in ice-cold URS. The composition of URS is 110 mM NaCl, 15 mM KCl, 15 mM $MgSO_4$, 0.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 7 mM glucose, 10 mM mannitol, 7.5 mM 2,3-butanedione monoxime, 0.5 mM EDTA, 10 mM Hepes, pH 7.5, , and 300 mOsm/kg water. For intermittent perfusion, the stored heart will be perfused via an aortic cannula with room temperature URS containing 6% hydroxyethyl starch saturated with 100% O_2 at a perfusion pressure of 60 mm Hg. Donor animals are euthanized under deep anesthesia as a result of cardiac donation and exsanguination.

Protocol for heart transplant survival surgery in canines. Healthy dogs will be housed in the Vivarium animal rooms upon arrival from the supplier. The animal will only be removed from the room on the day of the experiment.

Survival transplant experiments will be carried out in an aseptic animal operating room in the vivarium. The recipient dog will be anesthetized and ventilated as the donor. The animal will be initially anesthetized with intramuscular injection of 20 mg ketamine/kg and (0.6-0.8 mg atropine /kg. Then 25 mg sodium pentothal /kg will be delivered via cephalic vein catheter. After tracheal intubation and supported by mechanical ventilation, halothane maintenance anesthesia will be used thereafter (1.0-1.5 MAC). To assess surgical plane, we will monitor toe pinch reflex, corneal reflex, heart rate, and mucous membrane color every 15 min before surgery and during the first hour of surgery and every 30 min for the rest of the surgical period. The chest will be opened by a median sternotomy. After being fully heparinized, the animal will be put on cardiopulmonary bypass (CPB) and the native heart is excised along the atrioventricular groove leaving the atrial cuffs intact. Orthotopic heart transplantation will be performed using the methods originally developed by Lower and Shumway (61). When all anastomosis are done, cross clamp of the aorta will be released to start reperfusion. An isoproterenol drip (0.01-0.02 µg/kg/min) will be started. The implanted heart will be electrically defibrillated and electronically paced at 110 bpm. After a regular sinuous rhythm and a minimal stable mean arterial blood pressure of 70 mm Hg are reached, the recipient will be weaned off the bypass gradually. Protamine will be administered slowly to avoid hypotension. All pump blood will be returned to the recipient if possible. Inotropic support will be slowly weaned off 2 hours after the recipient is off bypass. Systematic arterial and venous pressures of the recipient will be recorded every 30 min for a period of 2 hours after off CPB using a computer based 8-channel MacLab data acquisition system. Chest closure will be performed with standard surgical technique. Air and blood are evacuated from the thoracic cavity via a chest tube, which is aspirated regularly following the operation and removed 24-48 h after surgery. Sternum closure will be performed with Tyton ties. The first layer of muscle will be closed with interrupted stitches using 2

chromic gut. Subsequent layers of muscle will be closed with interrupted stitches using 0 Chromic Gut. The subcutaneous layer will be approximated with continuous stitches using 2-0 Chromic Gut. Finally, the skin will be sutured with interrupted stitches using 2-0 Vetamid. After surgery, the animal will be moved to intensive care and maintained on mechanical ventilation until the animal will be able to breathe spontaneously. Ventilation will be discontinued and extubation will be performed.

Postoperative follow-up after cardiac transplantation. Routine postoperative procedures include 1 brief daily physical examination - rectal temperatures, 2. daily blood gas and chemistry samples for the first 2 days, 3. daily administration of immunosuppression regimens - cyclosporine, 4 mg/kg/day oral, methylprednisolone, 0.5 mg/kg/day oral, azathioprine, 2mg/kg/day oral, 4. wound dressings are changed 3 times a week. 5. echocardiography to monitor heart function – On day 1 and day 7 post-transplant and once a week thereafter. 6. Endomyocardial biopsy for verification of suspected rejection episodes (see detailed method below). 7. Treatment of rejection episodes (see detailed method below). 8. The LV end systolic and end diastolic cavity volumes will be measured using Simpson's Rule from 2D echocardiography. LV diastolic function will also be determined by mitral valve Doppler echocardiography. LV ejection fraction (LVEF) will be calculated from the end systolic and end diastolic cavity volumes. Fractional shortening of the LV cavity will also be reported. Cardiac output will be determined by thermodilution technique.

The recipient dog will be kept alive for a period of 3 months. At the end of the expériment, the animal will be anesthetized with iv injection of pentobarbital (50 mg/kg). After establishing the surgical plane, the chest will be open with a midline sternotomy and the heart will be cardioplegically arrested and excised for histology and biochemical samples. The animal will be euthanized due to exsanguination.

Written records of anesthetic parameters, surgical procedures, and drug administration will be recorded chronologically (See attachments for Sample forms). The animals will experience minimal discomfort during the initial induction of anesthesia due to intramuscular injection. This minimal pain will be of extremely short duration of one minute or less. No adverse physiological, pathological, or behavioral sequelae are expected. The donor animal will not experience pain or stress other than already described above. The recipient animal will experience post-OP pain from heart transplantation surgery. It will be expected to experience stress due to daily check up, drug administration for immunosuppression therapy, blood sampling, occasional endomyocardial biopsy for suspected rejection and antirejection or antiinfection treatments.

Endomyocardial biopsy procedure. The dog's right neck will be prepped and draped in the usual sterile fashion. Under ultrasonographic guidance, a 6 Fr 11 cm sheath will be placed in the right internal jugular vein using a modified Seldinger technique. Under echocardiographic and fluoroscopic guidance, a 6Fr 1.8 mm Maxxim "Jawz" curved bioptome will be utilized to obtain 5 RV endomyocardial specimens. No complications have been noted after biopsy procedure by echocardiographic imaging. The dog will remain hemodynamically stable throught the procedure. The short sheath will be removed and adequate hemostasis will be obtained.

Grading and treating rejection. We will utilize the standard ISHLT grading system to grade rejection.

Grade 0 - no rejection
Grade 1A - mild acute rejection
Grade 1B - mild acute rejection
Grade 2 - focal moderate acute rejection
Grade 3A - multifocal moderate acute rejection
Grade 3B - diffuse moderate rejection
Grade 4 - severe acute rejection

For anti-rejection treatment, immunosuppressives will be increased for Grades 1B and 2. We would pulse steroids for hemodynamically significant Grade 2 and up and treat 3B and 4 aggressively with polyclonal antibodies or more. We also look for humoral rejection using Cd4 staining. Antirejection episodes will be treated by raising methylprednisolone to 15 mg/kg/day (iv) and raising the azathioprine dose by 2.5 mg/kg/day (IV).

Protocol for procurement of vervet (*Chlorocebus aethiops sabaeus*) hearts. Fully adult (~7 kg) vervets of either sex will be sedated with 10 mg/kg ketamine and 1 mg/kg xylazine and then anesthetized with 1-5% isoflurane in 100% oxygen according to established procedures at the Behavioural Sciences Foundation in St. Kitts, B.W.I. A mid-ventral laparotomy will be performed and a region of abdominal aorta will be prepared for cannulation. A large bore cannula containing URS will be introduced into the abdominal

aorta, advanced to the aortic arch with the chest closed, and firmly ligated in place. The animal will be ventilated and heparinized (1000 units/kg), and a median sternotomy will be performed with electrocautery to prevent bleeding. The aortic arch and the superior and inferior vena cava will be identified. The vessels branching from the aortic arch will be clamped and the superior and inferior vena cava will be severed. The heart will be allowed to beat approximately five times to empty itself of intraventricular blood, and perfusion will then begin at 110 mmHg with ice-cold URS. This procedure should induce rapid cardiac perfusion and cooling, and cardiac arrest should occur in about 20 seconds. Perfusion then continues until the balance of 1 liter of URS is expended. During this time, blood is suctioned from the chest and the pulmonary artery and vein are identified and severed to ensure adequate ventricular drainage and prepare the heart to be removed. At the end of in situ perfusion, the heart is excised, rinsed free of blood using topical sterile normal saline, and transferred into 500 ml of ice-cold URS in a sterile shipping container surrounded by ice. The ice bags are sealed shut using a triple bagging system to preclude leakage during shipment, the package is sealed, and shipping is done using transport-safe packaging and plenty of cushioning to preclude impact damage to the heart.

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LE Magazine January 2006

Transplanting Cryopreserved Kidneys

Dr. Gregory Fahy, 21CM's chief scientific officer, is the world's foremost expert in the vitrification of biological systems. Dr. Fahy has been working for decades to cryo-preserve kidneys well enough to transplant them successfully, first at the American Red Cross in Maryland and now at 21CM in California. The 21CM lab is the only one in the world currently pursuing vitrification of large tissue masses. Another key scientist working at this lab is Dr. Brian Wowk, who has developed the world's first practical ice-blocking compounds, which are used with combinations of cryoprotective chemicals in 21CM's vitrification solutions. These solutions allow tissues to be cooled to extremely low temperatures without the formation of ice. At about -123° C, these tissues are transformed into a glass-like state, which differs dramatically from the crystalline state that occurs during freezing. Vitrification stops life processes cold, without the structural and biochemical damage caused by ice crystals. (Fig. 8.)

Survival of the First Vitrified Kidney

At the annual meeting of the Society for Cryobiology in July 2005, Dr. Fahy announced the survival of a rabbit that received a transplanted kidney after the organ had been vitrified to -130° C and then re-warmed. This result was based on the vitrified kidney's ability to provide sole renal life support in the recipient animal until it was removed for examination 48 days after it was transplanted. The transplanted kidney sustained some damage from ice formation in its center, but that damage was entirely absent in other parts of the core of the kidney, enabling the organ to support life in a normal fashion.

This remarkable achievement is a major medical milestone that moves us closer to establishing banks for the long-term cryopreservation of human organs. This research is currently being written up for publication in a scientific journal, while further research to perfect the vitrification of rabbit kidneys continues. In 2004, Dr. Fahy and colleagues published a paper reporting the survival of eight rabbits that had received re-warmed transplanted kidneys that had been cooled to -45° C. This breakthrough was based on earlier advances involving the ability to control cryoprotectant toxicity, nucleation, ice crystal growth, and chilling injury.[10]

21CM has also had success in developing new methods for short-term storage of kidneys and hearts, as well as vitrification of cartilage and corneas. It has been particularly successful in vitrifying corneas, which cannot be frozen effectively. Corneas are complex structures in the eye that often become opaque with advancing age, which can result in blindness. Corneas vitrified by 21CM scientists generally show no cell loss or cell death, and remain transparent after transplantation.

Vitrifying Brains and Whole Organisms

The most ambitious research being performed at the 21CM laboratory involves experiments to vitrify rabbit brain slices, entire rabbit brains, and entire rabbits. The short-term potential of this research includes the use of well-preserved brain slices to test potentially therapeutic drugs and antidotes to potential biological and chemical weapons of mass destruction. This research could also lead to the availability of well-preserved brain sections for neurobiology experiments and the treatment of brain diseases such as Parkinson's and Alzheimer's. This research is also essential for achieving suspended animation in humans, which could be instrumental in radically extending the life span of millions of people.

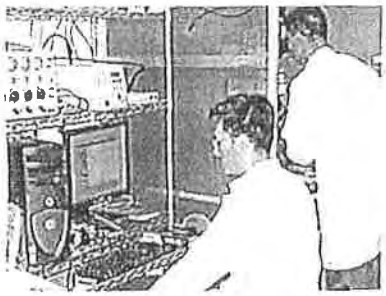

Figure 9. Joon Chang (left) and Dr Yuansheng Tan evaluating brain-slice electrical activity at 21CM's state-of-the-art recording station. Dr. Tan is observing a hippocampal brain slice through a microscope located in a Faraday cage that screens out environmental electrical signals while he inserts electrodes into the slice. Mr. Chang is monitoring the results of Dr. Tan's activities on a virtual oscilloscope.

Recovering Viability in Brain Slices

One test we have used to evaluate brain slice function after vitrification and re-warming is the potassium/ sodium ratio, or ion transport capacity. Another is to determine whether there is normal preservation of slice structure. These tests have shown apparently normal structure and function in hippocampal brain slices. The hippocampus is an area of the brain involved in memory consolidation, storage, and retrieval, and is the part of the brain most sensitive to oxygen deprivation. To further evaluate the effectiveness of its brain studies, 21CM has established an in-house neurophysiology laboratory to measure electrical activity in brain slices and sections. This lab is headed by Dr. Yuansheng Tan, who is assisted by Joon Chang. (Fig. 9.)

In initial studies, it was determined that vitrified, re-warmed brain slices were alive but electrically silent. After several refinements in technique, the 21CM brain slice team was able to achieve essentially normal electrical responses in up to seven of nine slices, which compares favorably to the rate of normal electrical responses in uncooled control slices not exposed to vitrification solutions, and exceeds the rate of electrical responses in uncooled control slices in many other laboratories. This is the first time electrical activity analogous to a normal electroencephalogram (EEG) response has been achieved in organized brain tissue after cooling to temperatures low enough to achieve vitrification.

Vitrifying the Entire Brain

Like studies of isolated hippocampal slices, studies of entire rabbit brains have found that all regions of the brain appear to be structurally preserved after vitrification and re-warming. Initial studies have shown that it is difficult to preserve the brain's ability to respond electrically after five hours of cold storage or cold perfusion in the absence of cryoprotectant chemicals. Studies are under way to overcome this problem, however, and the 21CM team will eventually look at electrical activity in brains that have been perfused with vitrifiable concentrations of cryoprotectants. It appears as though the entire brain can be vitrified, but further research is required to see whether normal electrical activity in the brain can be preserved after vitrification.

Vitrifying the Entire Body

When whole rabbits were vitrified, scientists collected tissue samples from many brain and kidney regions as well as from the heart, lungs, liver, intestine, muscle, skin, fat, stomach, and other areas. Using a device called a differential scanning calorimeter, scientists tested the samples to see whether ice formed within them. The differential scanning calorimeter can cool tissue samples to vitrifiable temperatures, warm them up, and measure how much ice has melted during warming. The amount of melted ice is equal to the total amount of ice formed during both cooling and warming, and the temperature at which the ice melts is a measure of how much cryoprotectant was in the tissue before it was cooled.

The results showed that the most difficult tissue to vitrify in the entire body was the center of the kidney, called the inner medulla. Most of the tissue samples showed only small amounts of ice formation, or none at all. Since 21CM is moving toward successful vitrification of whole kidneys, it appears that the entire body can be successfully vitrified as well. This suggests that whole-body

suspended animation is an achievable goal, but a great deal of additional research will be necessary to attain this goal.

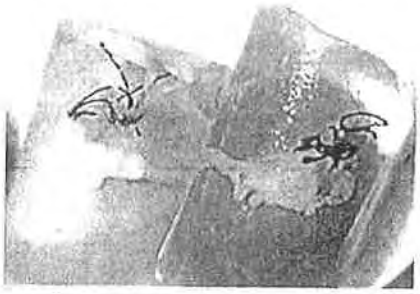

Figure 8 The kidney on the right was vitrified while the kidney on the left was frozen. There is massive ice formation inside and outside of the frozen kidney while there is virtually no ice in or on the vitrified kidney which looks like a natural kidney at normal body temperature. Both kidneys were photographed at -140° C