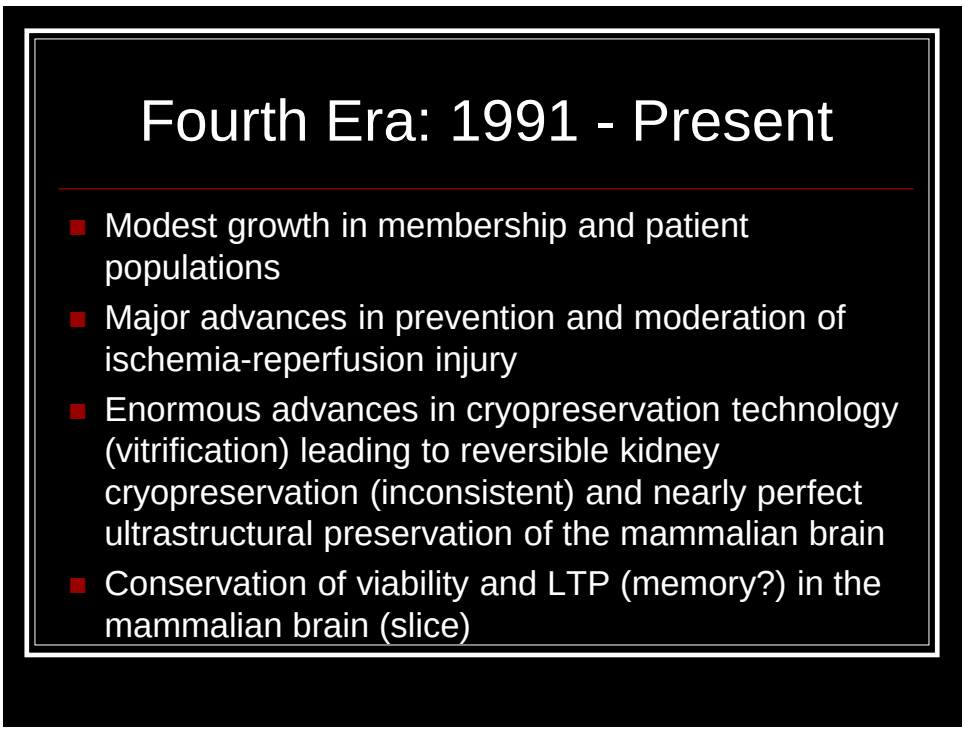
Cryonics: An Historical Failure Analysis

Part III: Dissociation of Researchers from the Clinical Environment

Mike Darwin

1991-2010

FOURTH ERA: 1991-PRESENT PARADOX



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As a result of the schism of Alcor in 1991 and the increasing isolation of basic researchers in cryonics from its clinical application, cryonics entered its Fourth Era which, arguably, it is still in. This phase has been characterized by sluggish growth in both membership and patient populations, stalled or deteriorating quality of patient care, while at the same time there has been a stunning (if paradoxical) growth in the available cryopreservation technology.

The enormous energy and enthusiasm with which cryonics was aggressively promoted from 1981 to 1991 has all but evaporated. When I consider the vastly improved

scientific and technological position that cryonics is in today, as compared to the 1980s, I can only shake my head in wonder at what could have been accomplished during that interval if we had had such comparatively solid scientific underpinnings. The advances that have been made have at come at great cost and effort and yet, ironically, they remain largely unexploited either in terms of promoting cryonics, or in terms of improving the care cryonics patients are receiving.

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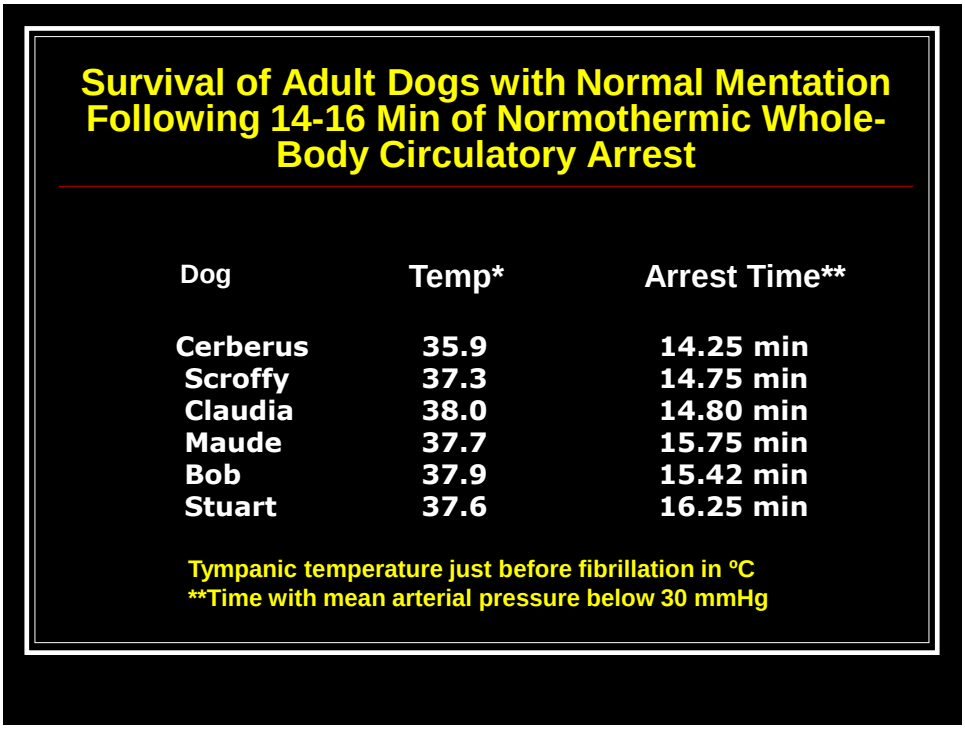
And the real tragedy and the real paradox in all of this is that finally, almost 50 years after its inception, cryonics has reached the point where its most formidable unknowns and technical stumbling blocks have been overcome. In

other words, never has there been a time when the available technology was more promising and solid than is now the case, and conversely (and paradoxically), never has the application of that technology to cryonics patients been more unapproachable.



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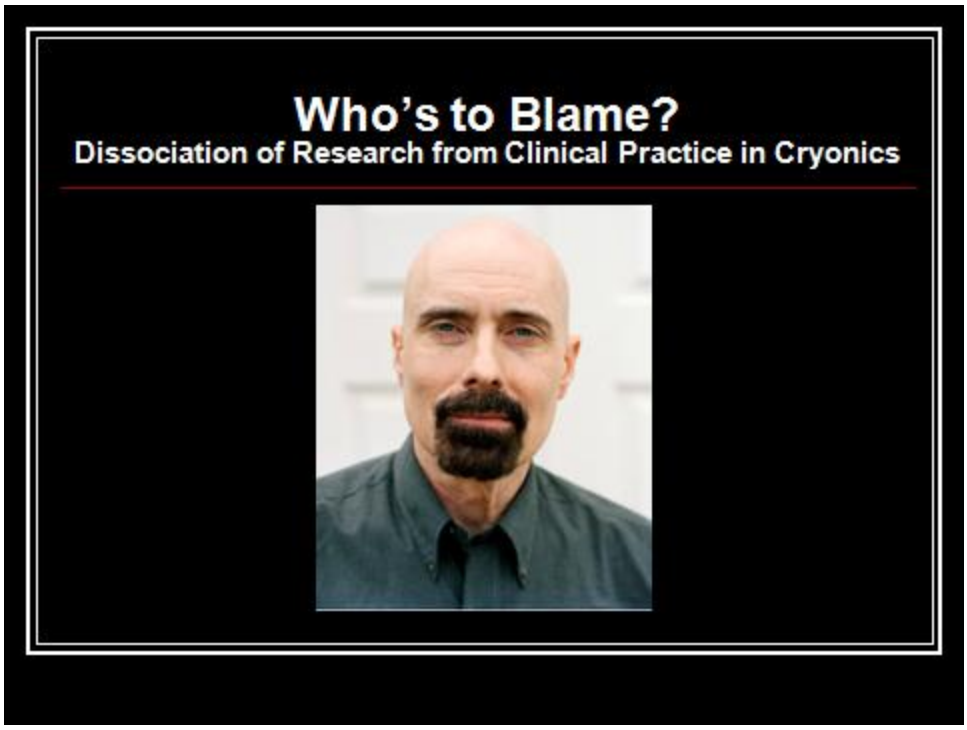
And the double irony is that most of these advances have been made by scientists who are themselves cryonicists! The work that my colleagues and I did at 21st Century Medicine demonstrated definitively that it is possible for dogs to survive ~16 min of normothermic ischemia and recover completely – without neurological deficit.

Thus, a major concern of both the public and the medical community over the perceived barrier represented by normothermic ischemia in excess of 4-6 minutes has been laid to rest. What's more, cryonics personnel at that time had complete mastery and control over the enabling technologies (semi-automated CPB, drug prep, novel medical-surgical interventions) because *they* developed them!

It is comparatively easy to summarize the progress made in mitigating ischemia-reperfusion injury (IRI) because the outcome is unequivocal: survival and normal mentation vs. coma and death. An extensive discussion of the mechanics of how this was achieved may be interesting, but is not essential (here) in order to appreciate that the advance has been made.



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And so it is at this juncture that it becomes my turn to accept responsibility for what may have been one of the most profound errors of judgment in the history of cryonics. If there is any one individual to blame for the dissociation of the clinic from the laboratory it is me.

It's a dangerous thing to say these words because they can just as easily be taken for hubris, when in fact they are a humiliating admission of the worst kind of short term, destructively self-interested decision making that I've been dissecting in these lectures. It is important that you understand not only that I am sincere in this belief, but also that you understand that I am *correct*.

After the schism of Alcor I found myself in a position I had not been in since I was, literally, a child. I was doing research more or less as a full time occupation without the tumult and distraction of cryonics, cryonics politics, and cryonics administration. I was also largely free from doing human cryopreservations (with all their attendant stress and heartache) since the majority of the highest risk members remained with Alcor after the split. For the first time in many years I was free to explore the scientific issues that interested me most – and this time, thanks largely to Saul Kent and the Life Extension Foundation, I had the truly priceless luxury of the money to do it with. As one unhappy employee at 21CM later barked at me: "Well, lucky you! This place is just your own private technological playpen, isn't it?"

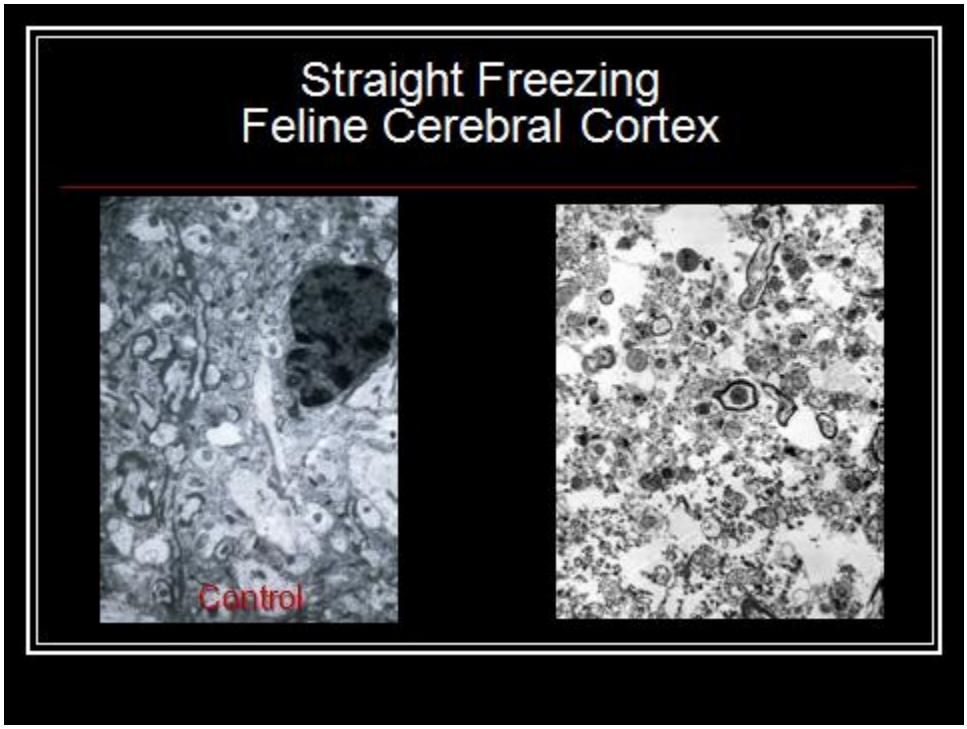
I wish I could stand here and say that that wasn't the case, but to a great extent it was. In the short term, at least from my point of view, there is no denying that this was a productive time for both me and, perhaps (in terms of the validating research on cryonics procedures) for cryonics as well.

But in the long term it was disastrous. While I have no way of knowing whether or not my labors to stick with cryonics and continue the work of creating that all important culture of professionalism would have been rewarded, I am certain, *absolutely certain*, that failing to *try* was a terrible mistake. I have searched my soul for the reasons why I made this choice, and I can find none that rise even slightly to anything approaching the level of a

valid excuse. I suppose I was part Dr. Faustus, part the sniveling coward, and certainly in large measure the fool who always believes there'll be another chance and that death would – if not make an exception in my case – at least grant me a sizeable extension of time in which to enjoy myself.

I cannot rewrite the past, but I can do what needs be done to help create a better future.

'QUANTIFYING' THE QUALITY OF CRYONICS OVER TIME



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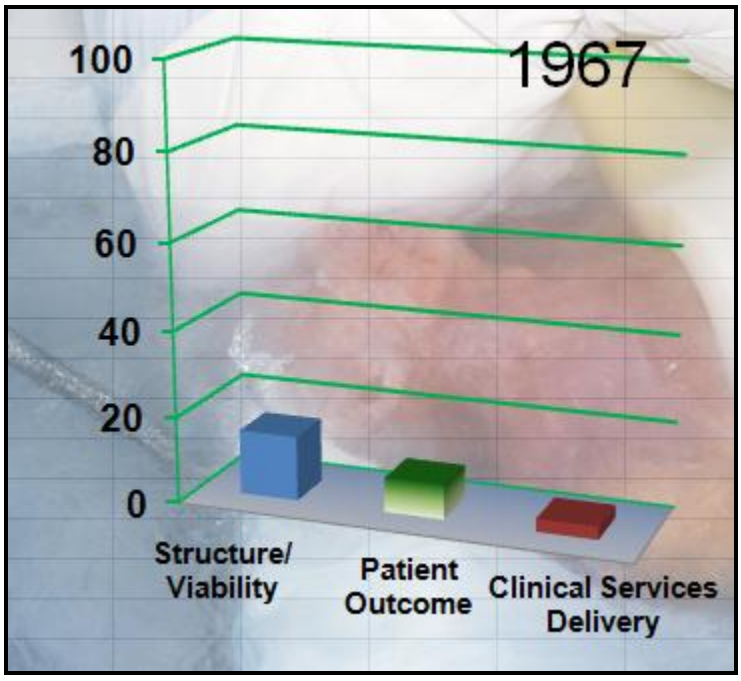
In the case of progress in cryopreservation over the past 40 years, we are still significantly short of being able to demonstrate recovery of intact mammals, or even of the

isolated brain following vitrification and cooling to deep subzero temperatures. In the absence of functional markers of viability we must instead rely upon the quality of structural preservation and surrogate markers for viability, where they are applicable.

For my own benefit in charting progress in this area I developed a scoring system based on ultrastructure following cryopreservation, rewarming and fixation. I won't pretend to argue its merits except as an approximate yardstick for measuring progress.

At left is brain tissue from a healthy animal perfused with fixative while under anesthesia and with no ischemia (e.g., beating heart). The fine architecture of the tissue is beautifully displayed and at 9K magnification it is possible to see intracellular organelles, such as the nuclei and mitochondria, as well the myelin sheathing, cell membranes and axoplasm.

At right is tissue taken from the same anatomical area of the cerebral cortex following straight freezing and rewarming. Cell membranes are no longer visible and the field of view appears more like a tissue homogenate than a section of brain tissue. I assign this level of injury a score of 15, as opposed to a score of 100 for control brain tissue. It is not possible to score higher than 75, regardless of the quality of ultrastructural preservation, in the absence of viability; viability therefore counts for 25 points.



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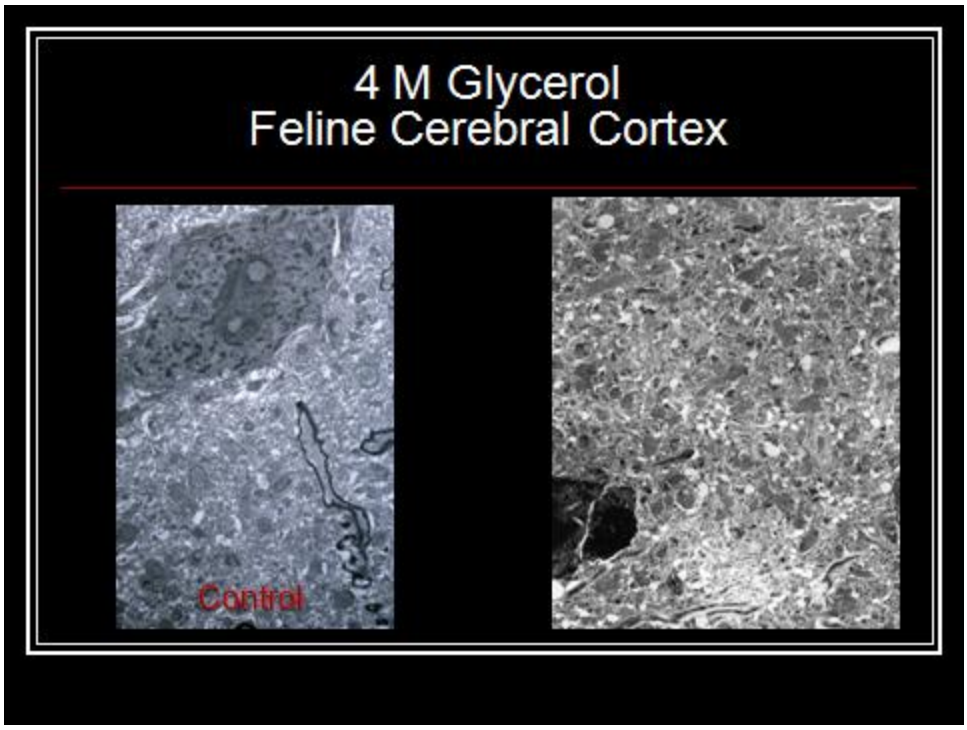
It is possible to do the same kind of scoring with respect to the pre-cryopreservation parts of cryonics procedures, as well. A patient with no peri- or post-arrest ischemia who was provided with immediate and effective CPS followed by CPA perfusion within the window of demonstrated viability (i.e., 5 hours of asanguineous perfusion at 5^{o}C) would thus get a score of 100 in terms of Clinical Services Delivery. In other words, that patient would have received the best technologically available pre-cryoprotective care it is currently possible to deliver.

Since all cryonics patients currently must be pronounced legally dead before the procedure can begin, no patient can score 100. I have arbitrarily decided that each minute of normothermic ischemia up to 5 minutes will count for the loss of 1 point. Thereafter, I have used other,

more generous criteria for adjudging the adequacy of care which I will not detail here. Suffice it to say that if the patient gets cryopreserved under conditions where cryoprotection is not possible, the score is 3 points.

If we go back to Dr. Bedford, the first patient cryopreserved in 1967, we see that the best possible score he could have achieved had he been immediately straight frozen after pronouncement was 15; but because of grossly inadequate post-arrest and pre-freezing care, his Patient Outcome Score is only 8. You'll have to decide for yourself how reasonable my scoring system is as we go along. But keep in mind; it is designed to serve only as a relative indicator, not as an absolute measure of performance, nor of patient recoverability.



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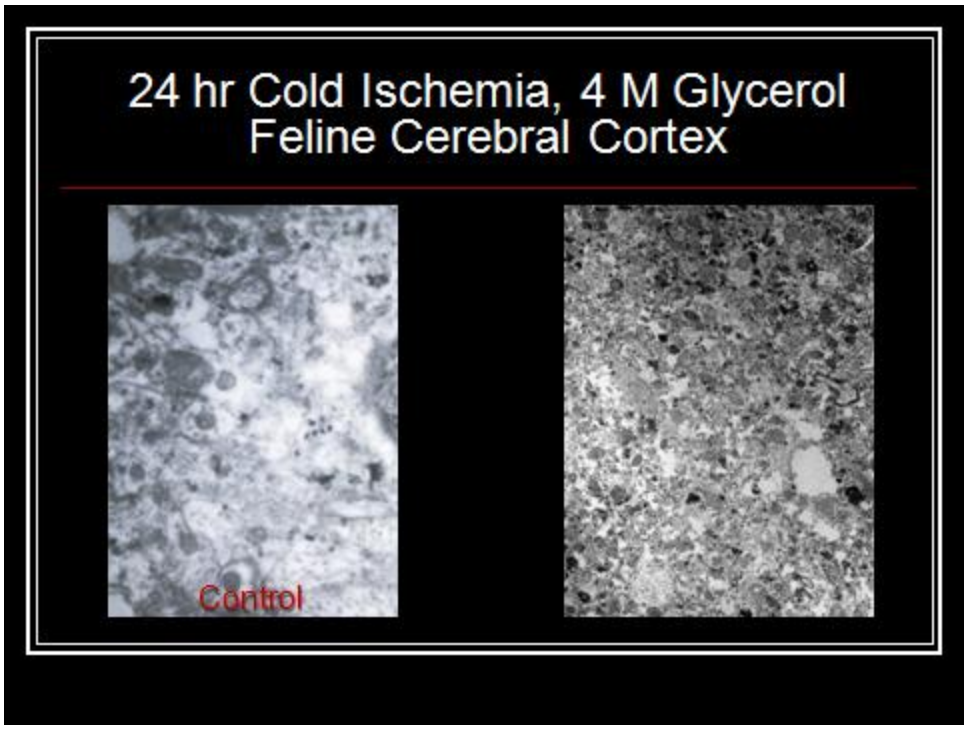
By 1978 we had progressed in cryonics to the extent that we understood the need to deliver at least 3 M of colligative cryoprotectant (usually glycerol) and we had finally actually developed (and learned to reliably apply) rapid (1-2 min) post-arrest mechanical external CPS. At left you see normal control brain ultrastructure, and at right you see what the gray matter of the cerebral cortex looks like after cryopreservation in the presence of 4 M glycerol. There is substantial mechanical disruption from ice and there is apparently considerable loss of detail in the ground substance of the tissue (the molecular fabric of the cells). I assign a score of 35 to brains cryopreserved in this manner.



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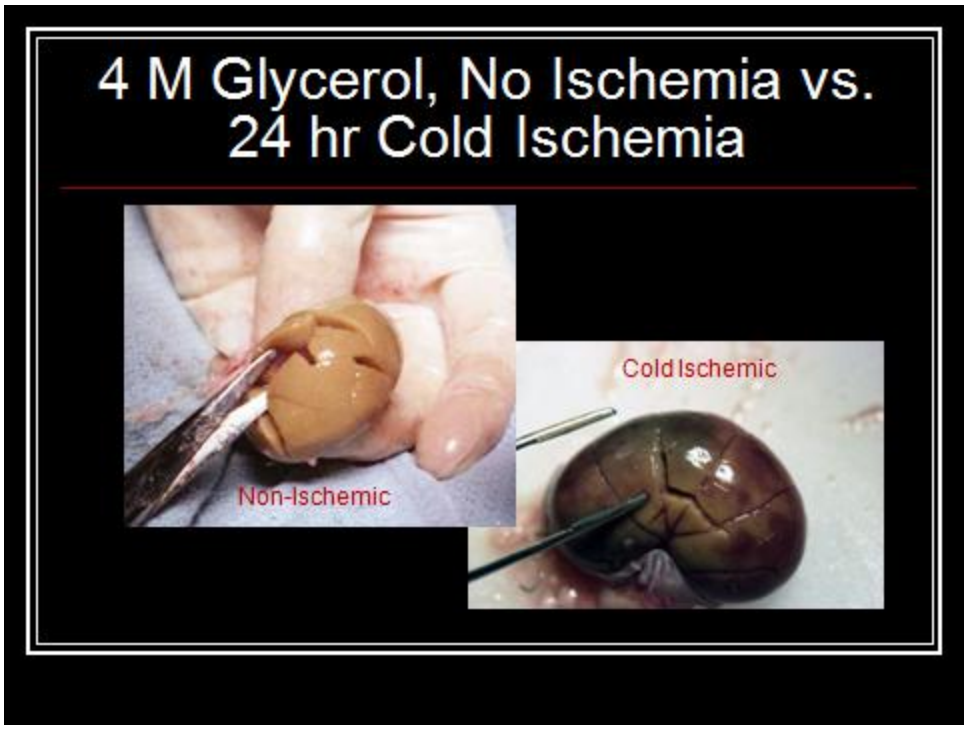
However, it is very important to realize that the results seen in the previous slide were obtained under ideal laboratory conditions with no period of peri- or post-arrest ischemia. If a period of warm and cold ischemia often experienced by cryonics patients (say, 15 minutes of warm ischemia followed by packing ice for 24 hours) is imposed before cryoprotective perfusion and deep cooling, the results are dramatically worse. Under these conditions the best possible score is 25.



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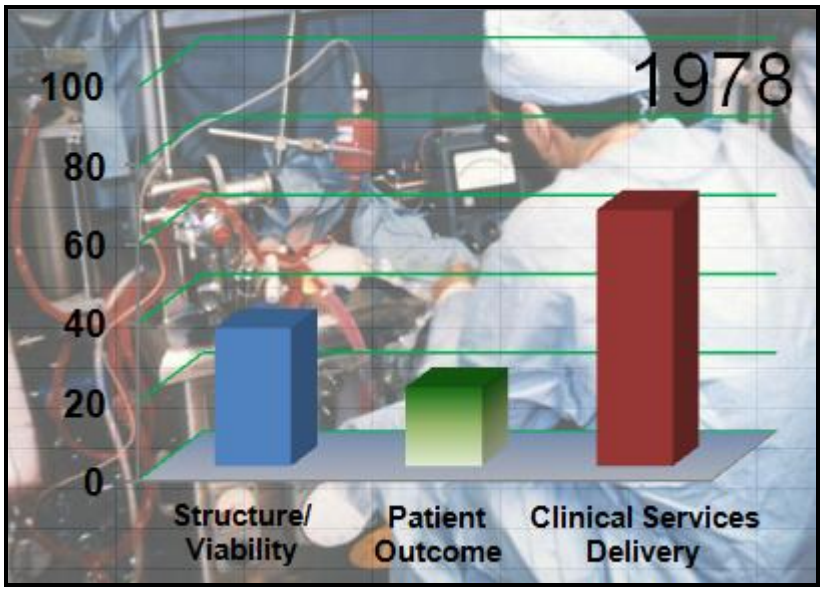
One of the reasons for the vast difference in structural preservation between ischemic and non-ischemic animals is the impact prolonged ischemia has on the vascular accessibility of the CPA. The kidney at the top left is from an animal perfused with 4 M glycerol in the absence of ischemia. The renal cortex is uniform in color and free from blood. The ‘cuts’ in this kidney are actually fractures that resulted from cooling the animal below the glass transition point (Tg) of the glycerol-water solution in the tissue. By contrast, the kidney on the lower right is from an animal subjected to 15 minutes of normothermic ischemia followed by packing ice for 24 hours. Cryoprotective perfusion was carried out in a manner identical to that used for the non-ischemic animals. Instructively, the terminal

central venous glycerol concentrations in all of these animals, ischemic and non-ischemic, were the same.

However, it is only necessary to look at the kidney from the ischemic animal to realize that blood washout was far from complete, despite nearly 90 minutes of continuous perfusion. While the venous effluent indicated equilibration to 4 M glycerol, it is likely that the volume of tissue in the ischemic animals in true equilibrium with the terminal concentration of perfusate (4M) was considerably less than 100%. It is also worth noting that the non-ischemic control animal kidney is more extensively fractured than the ischemic kidney. This is also an indication of poor glycerol equilibration.



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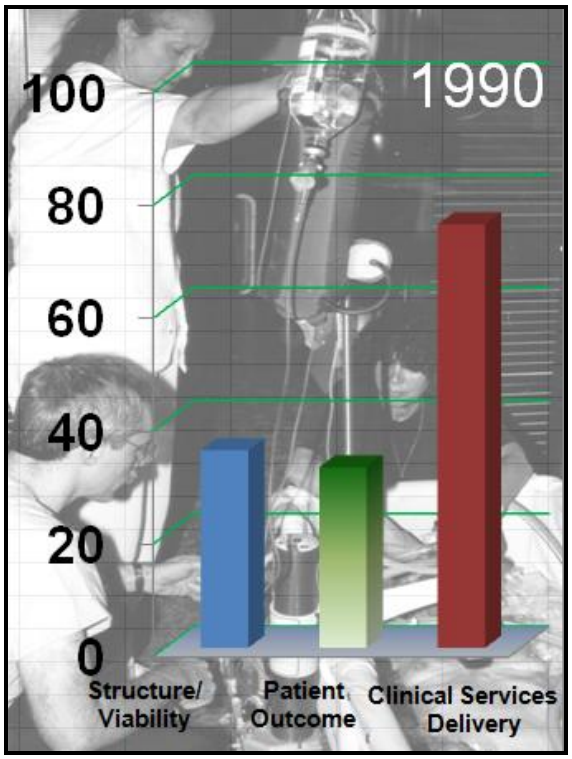
Returning to the real world of cryonics as it was *best practiced* in 1978; the best possible outcome was probably

in the range of 25 to 30 points. A major limiting factor at that time was the lack of availability of rapid post-arrest supportive cardiopulmonary bypass and the long time from cardiac arrest until the start of cryoprotective perfusion: typically in the range of 4-6 hours. External, closed-chest CPS is not effective at supplying adequate flow and external cooling is very slow under such conditions. As a consequence patients could be expected to suffer substantial warm/cold ischemic insult during this interval.



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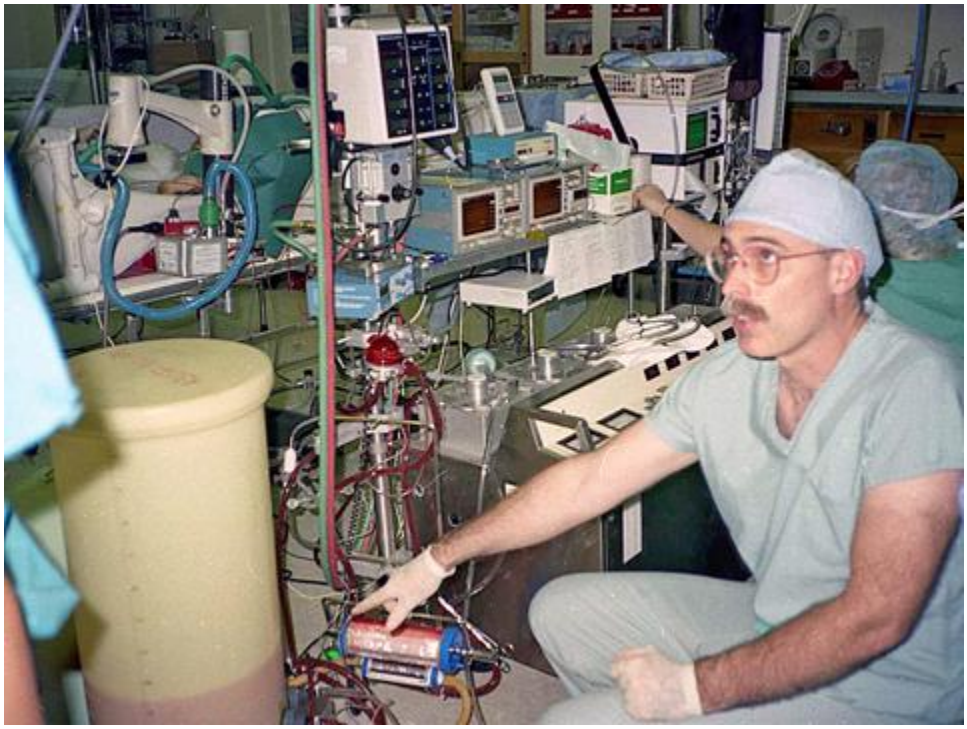
By 1990 substantial advances had been made in improving the efficacy of closed chest CPS and the first generation of truly effective cerebroprotective medications

was on-line. Additionally, the capability for initiating extracorporeal support in the home had been developed allowing for the initiation of bypass within 30 to 60 minutes of cardiac arrest. As a consequence of these improvements in Clinical Services Delivery patients arresting with a Standby Team in place came very close to reaching the maximum possible Patient Outcome Score (as determined by the best possible Structure/Viability Score) of 35.

WOKING FOR IMPROVEMENT



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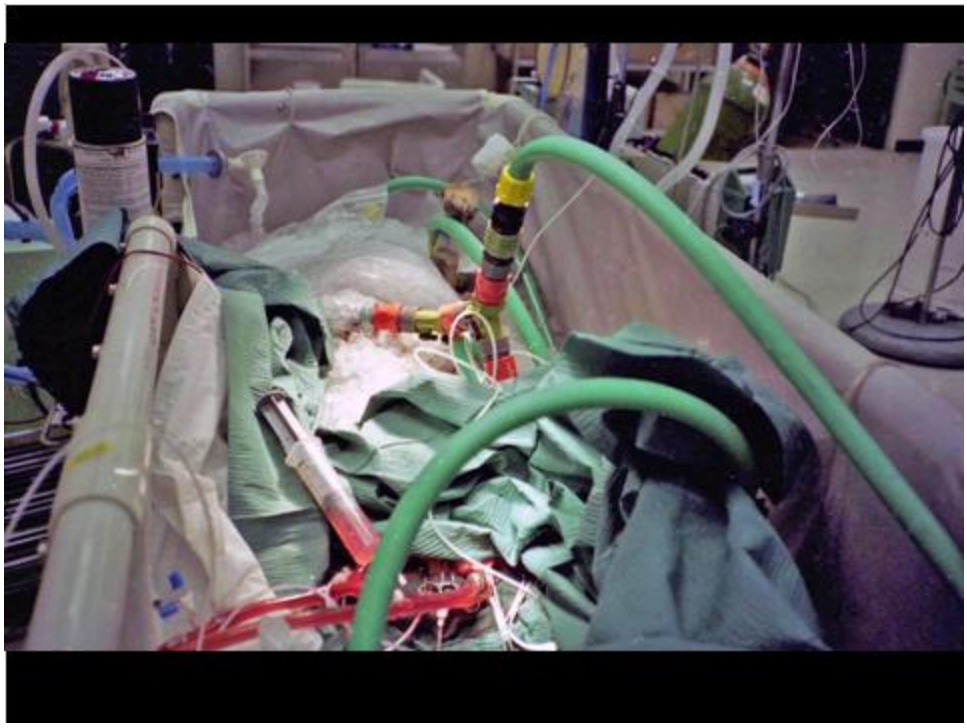
By the early 1990s I was anxious to push the limits of cryoprotection possible with glycerol and we undertook a series of experiments on dogs simulating a ‘typical’

cryonics case by imposing 5 minutes of post-arrest normothermic ischemia prior to initiating closed chest mechanical CPS.

My intention was to simulate, as closely as possible, the ‘ideal’ conditions under which actual human cryopreservations take place. If you look to the upper left corner of this slide you will see the PIB containing one of the experimental animals in this series. The ‘Thumper’ mechanical CPR device is visible but is not in use since cardiopulmonary bypass and cryoprotective perfusion are already underway.



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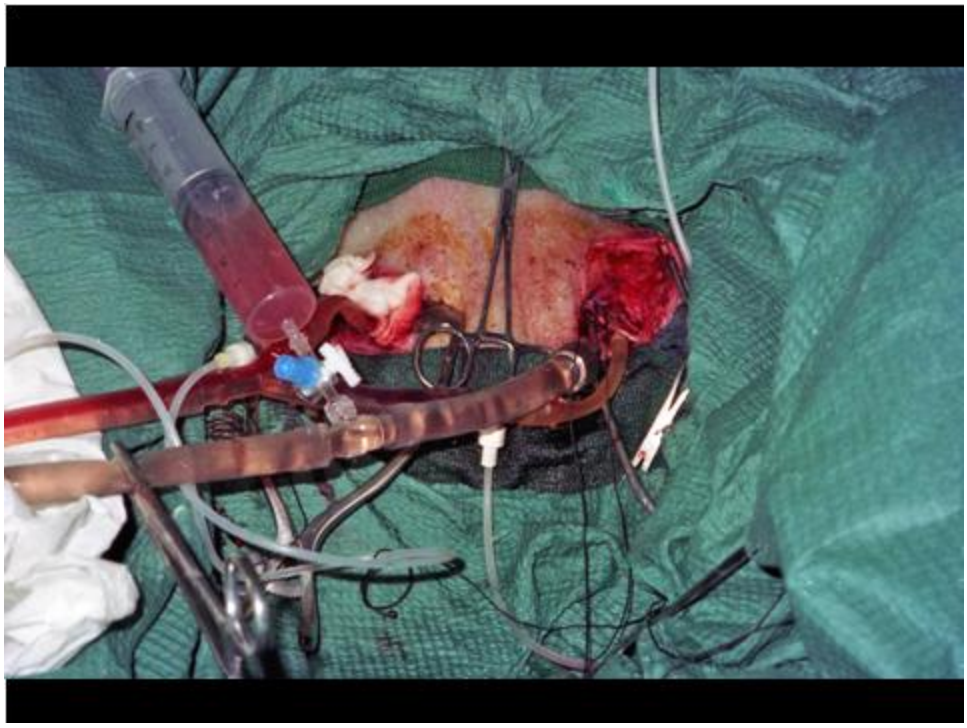
These experiments were also invaluable training tools since, as you can see, they employed the same equipment and procedures used in human cases.



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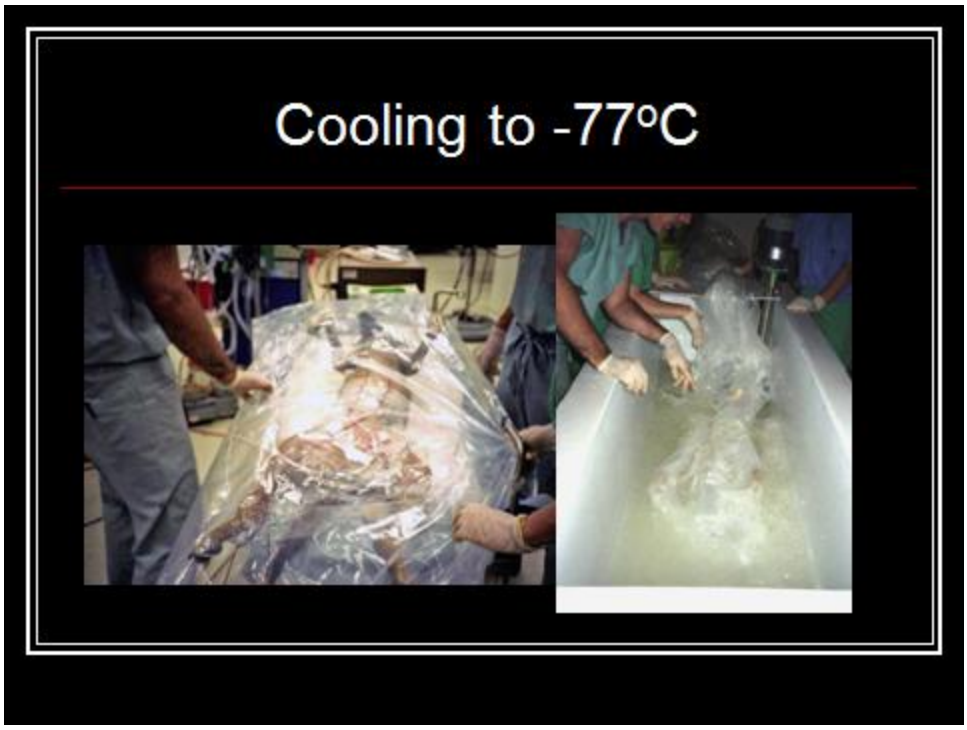
The animals were supported on closed chest CPS until CPB was initiated and they were then perfused with linearly increasing concentrations of glycerol terminating at a concentration 7.5 M of glycerol in the brain.

One of the things we learned from these experiments was that with the proper cannulae in an animal with only a brief (~5 min) ischemic insult it is possible to carry out cryoprotective perfusion of the entire body using the femoral vessels instead of performing a median sternotomy. This technique was subsequently used to greatly shorten the cold ischemic time in two human cryopatients by using the femoral cannulae that were already in place for extracorporeal support and blood washout during Transport.



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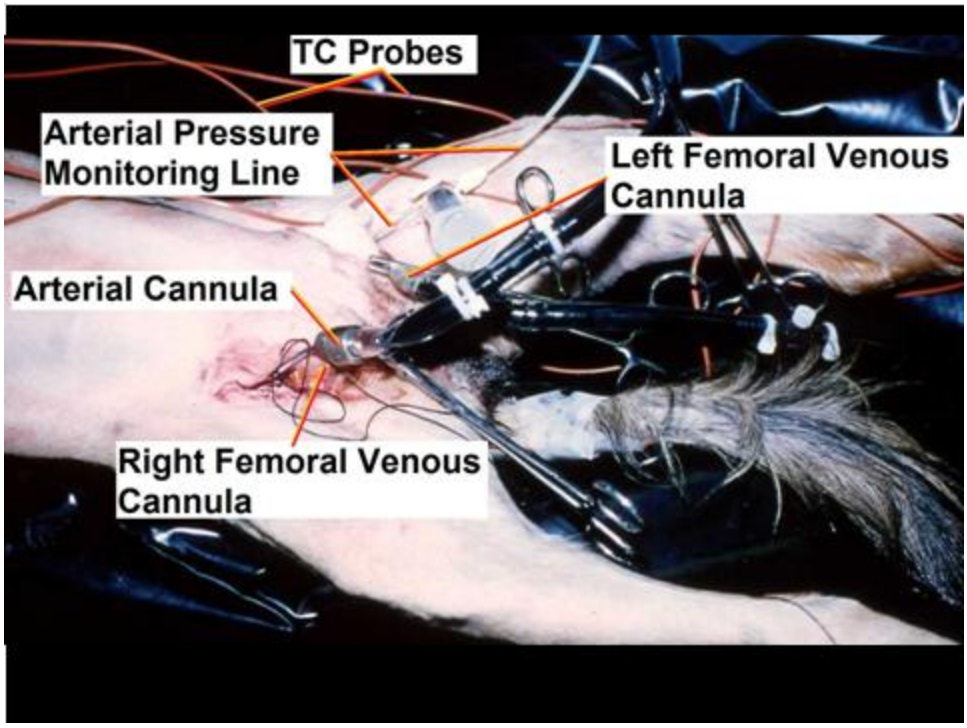
One important way in which these experiments did not map the experience of human cryonics patients was that we stopped cooling at -90°C, ~10°C above the glass transition point of the 7.5 M glycerol perfusate, in order to eliminate any chance of fracturing that would prevent reperfusion and fixation of the animals after thawing.



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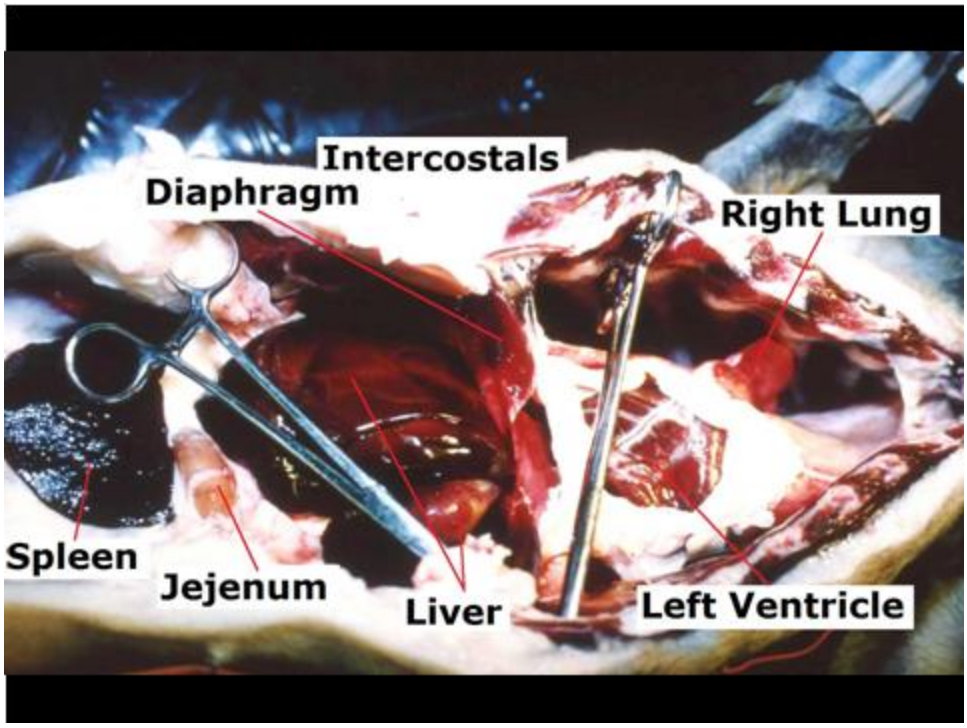
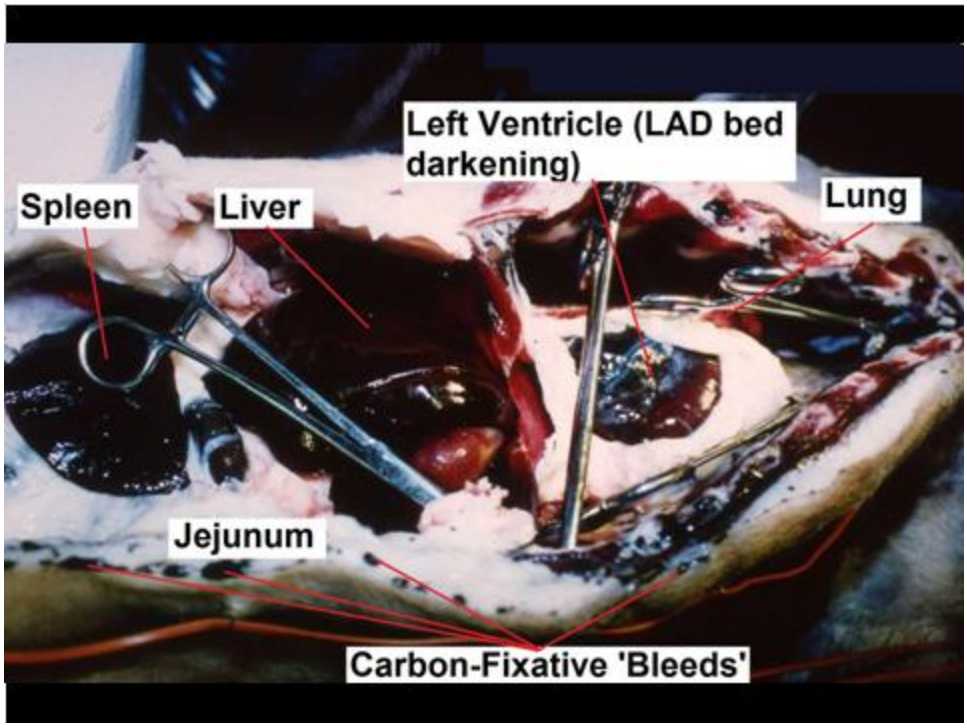
The animals were reperfused after thawing with fixative solution containing both 7.5 M glycerol and a suspension of microscopic (0.5 -1.0 microns) carbon black particles.



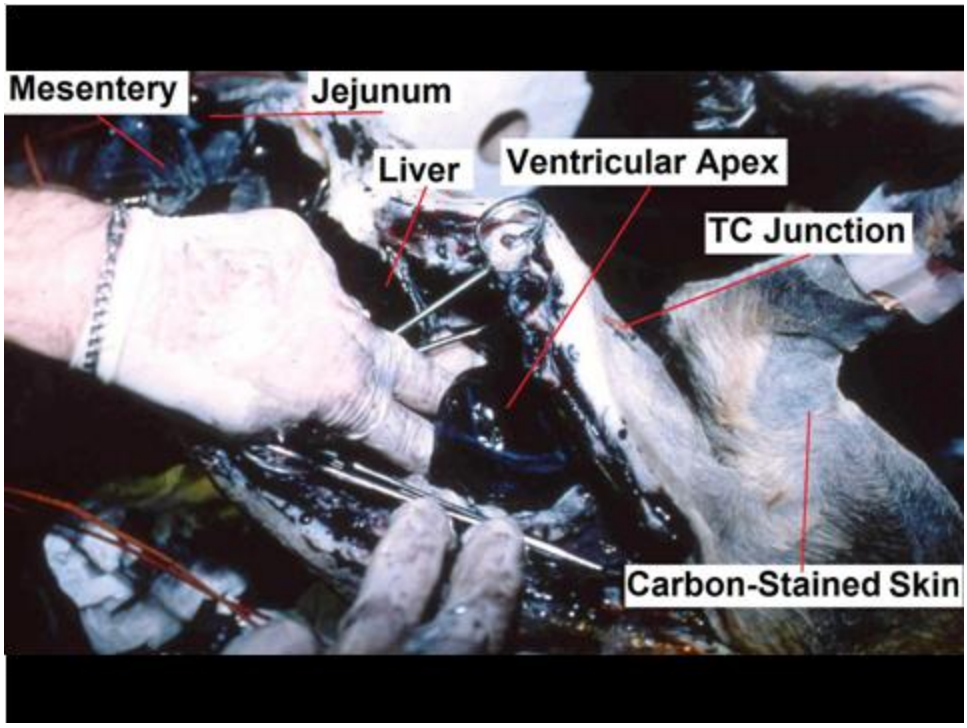
SLIDE 171

Before reperfusion was initiated we performed a mid-ventral thoracotomy and laparotomy to allow visualization of the thoracic and abdominal viscera.



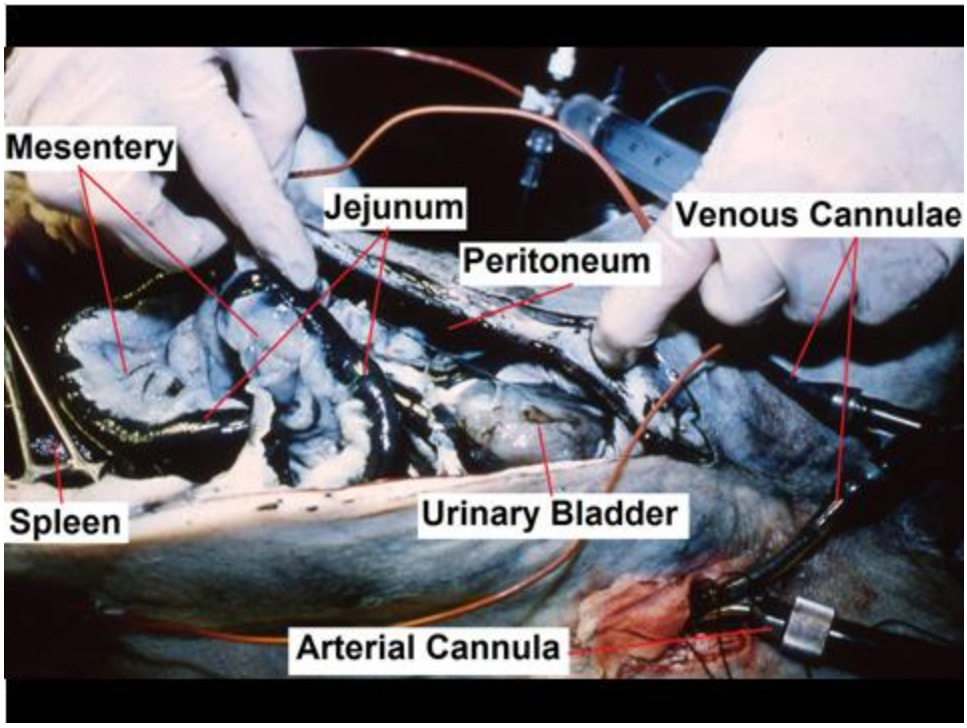
SLIDE 172

As you can see, carbon staining of the tissues occurs – providing they have an intact microcirculation. From many previous investigations we learned that carbon reperfusion is very poor in frozen-thawed tissues – even with concentrations of glycerol as high as 3 M.



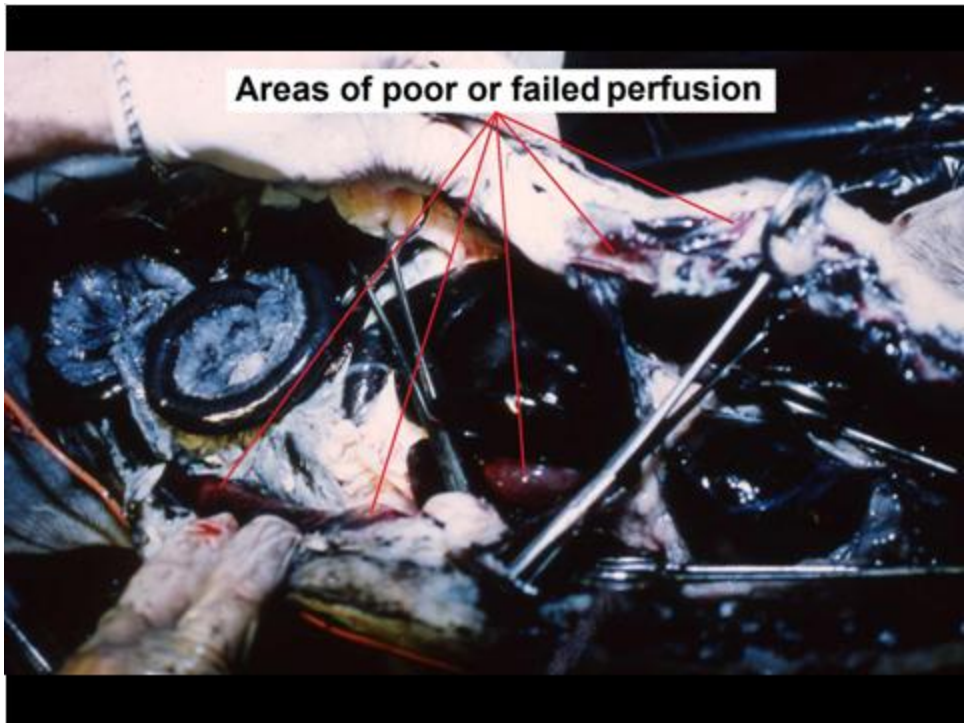
SLIDE 173

Carbon distribution (and accompanying fixative) was very good in these animals – by the far the most uniform and complete that we had observed.



SLIDE 174

Even poorly circulated tissues such as the urinary bladder and large bowel reperfused uniformly and completely.



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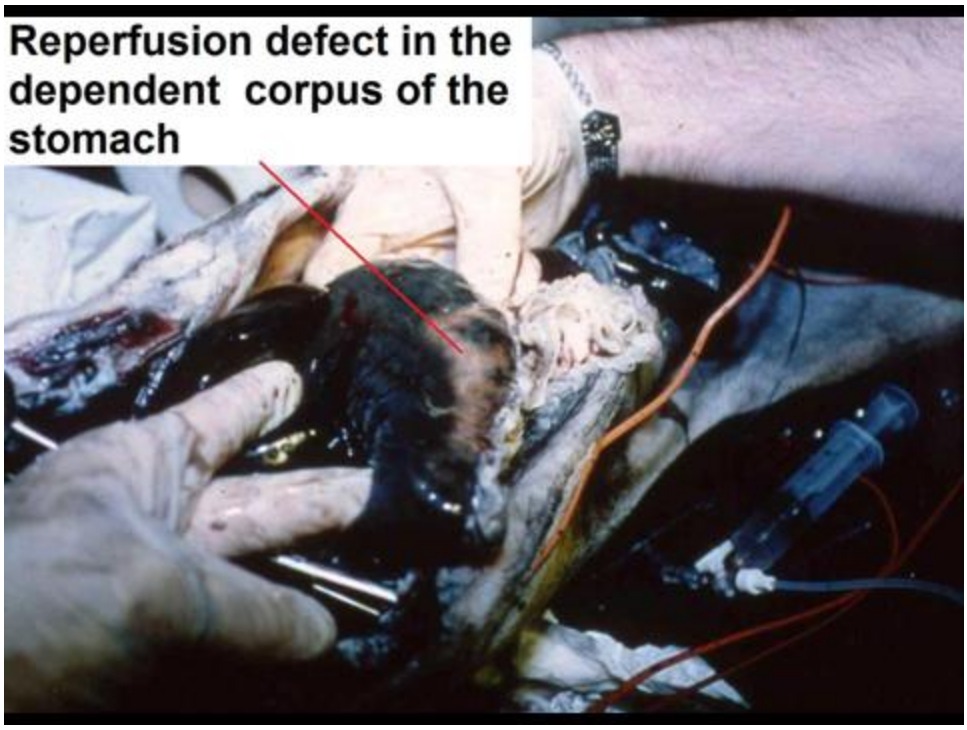
In all of the animals in this series (with the exception of the controls) there were a few scattered areas of failed reperfusion, but no consistent pattern was identified, with two exceptions.



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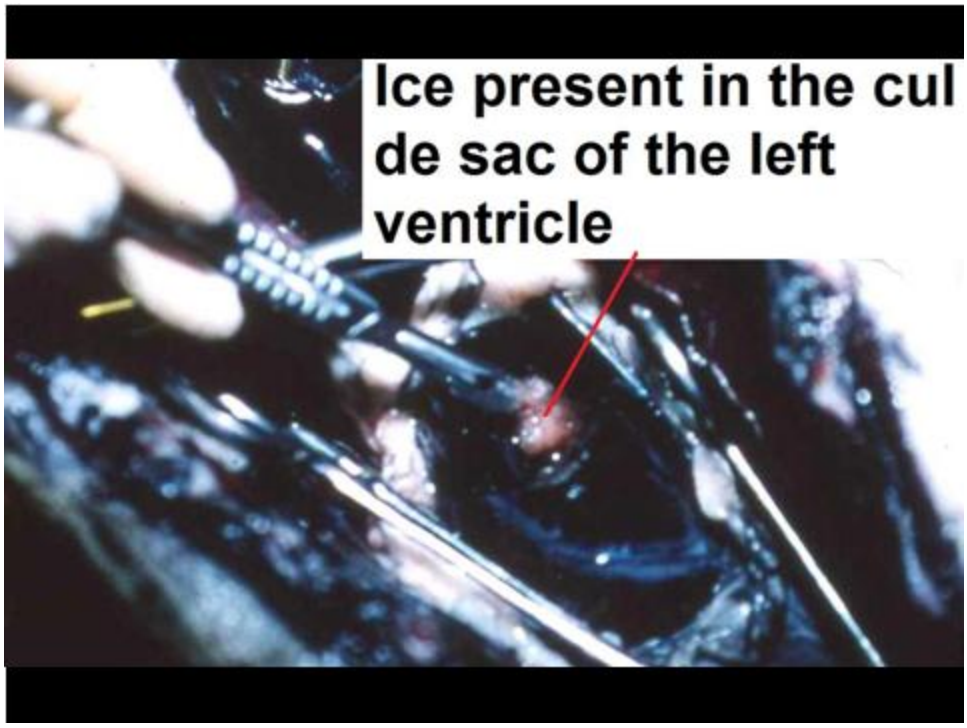
In animals that had solid food (kibble) in their stomachs the area underlying the kibble failed to reperfused – not just the gastric mucosa but the serosa as well. Apparently water contained in the kibble diffused out into the stomach wall and diluted the CPA to a point where capillary destroying amounts of ice could form.



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A similar phenomenon was observed with respect to the endocardium of the left and right ventricles where blood –tinged ice was observed to be still present following the conclusion of fixative perfusion! We began fixative perfusion when the animals thawed out which was ~ -6^{o}C; and the core temperature of most of the animals was still -1 to -2^{o}C at the end of fixative perfusion. The measured glycerol concentration in the intraventricular ice was ~5%.

In the future, as we hopefully approach fully reversible vitrification or minimally injurious vitrification, it will be necessary to vent the ventricles during CPA perfusion to prevent serious freezing injury of the endocardium.

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A wholly unexpected but not surprising observation was that the skin under the massager pad of the ‘Thumper’ CPR device failed to fully equilibrate with CPA. The blue arrow points to a pale area of skin overlying the distal third of the sternum which was directly under the Thumper massager pad during the period of closed chest CPS preceding CPA perfusion. Since the thumper piston was removed from the chest immediately after the start of extracorporeal support, the cause of the observed perfusion defect must have been as a result of the trauma associated with mechanical chest compression.

This observation would prove helpful years later in explaining areas of failed or inadequate perfusion in cryopatients undergoing vitrification protocols.

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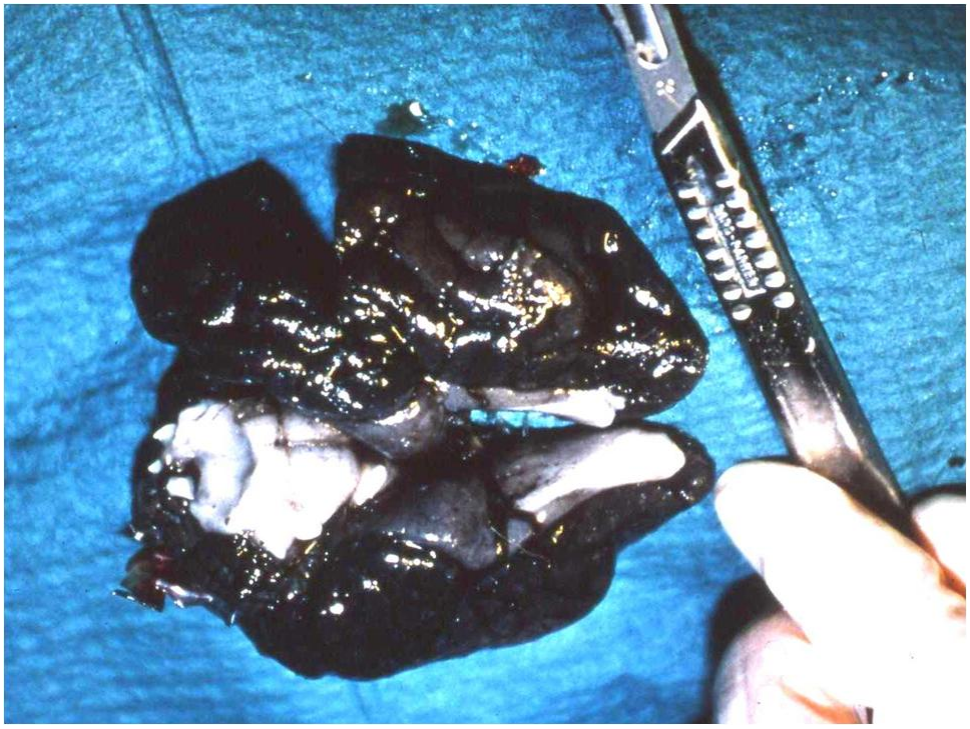
A serious concern had been whether the cerebrospinal fluid (CSF) surrounding the brain and contained within the cerebral ventricles would similarly act to dilute cryoprotectant in the brain parenchyma following the conclusion of perfusion, again resulting in severe injury. We found that this was not a problem, at least not in healthy young animals.

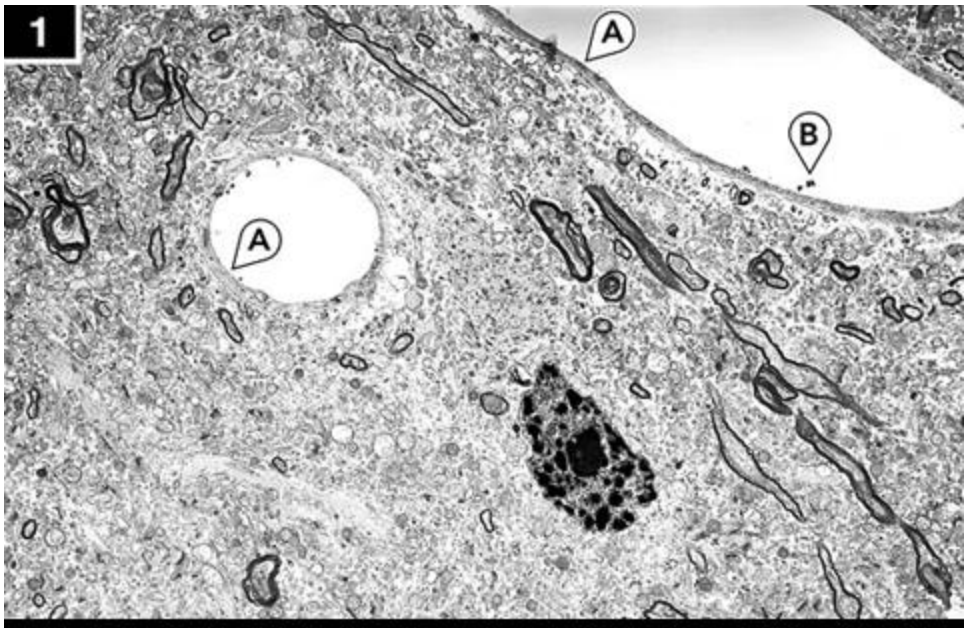
However, in the case of elderly humans with greatly enlarged ventricles due to cerebral atrophy, CSF dilution of CPA in the brain parenchyma may be a very real source of injury. Similarly, patients with space occupying lesions of the brain which are poorly perfused, such as hematomas, some brain tumors, and large diameter cysts secondary to parasitic or infectious disease will likely require

decompressive carniotomy, or extended duration CPA perfusion.



SLIDE 180

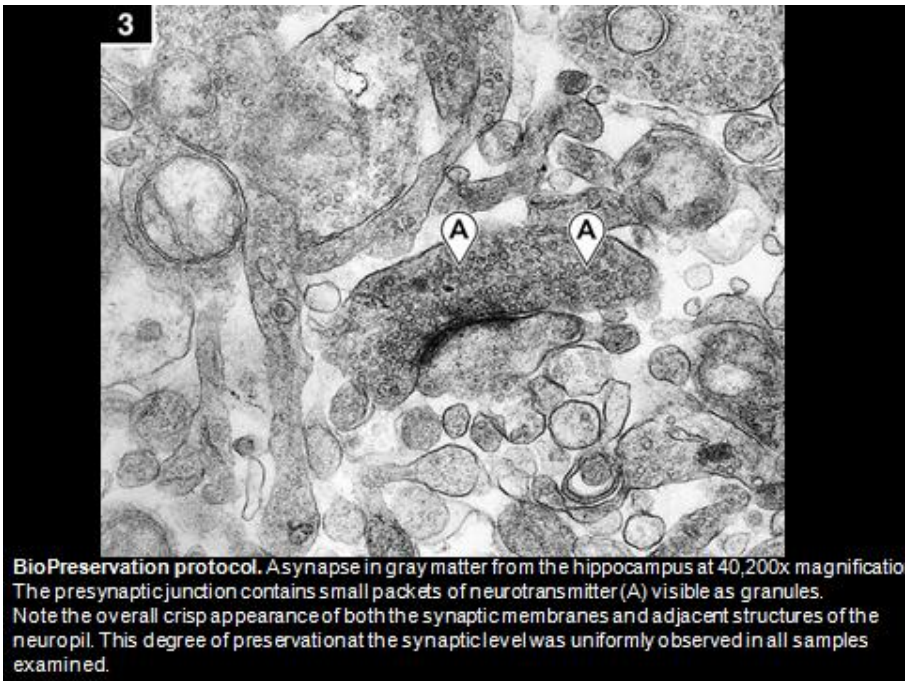
Overall, carbon reperfusion of the brains of these animals was excellent to good. Some animals exhibited relatively pale areas of the cerebral cortical surface as can be seen in the previous slide – but there were no instances in which a frank failure of reperfusion occurred.



BioPreservation protocol. Typical appearance of gray matter at 6700x magnification. Note intact capillary endothelial cells (A) and particles of carbon (B) in the capillary lumens. The overall appearance of the neuropil and of the axons and neurons is excellent.

SLIDE 181

By far the most important result of this work was that, for the first time, it was possible to achieve the breathtaking ultrastructural preservation you see in this slide and in the next one.



BioPreservation protocol. A synapse in gray matter from the hippocampus at 40,200x magnificatio
The presynaptic junction contains small packets of neurotransmitter (A) visible as granules.
Note the overall crisp appearance of both the synaptic membranes and adjacent structures of the neuropil. This degree of preservation at the synaptic level was uniformly observed in all samples examined.

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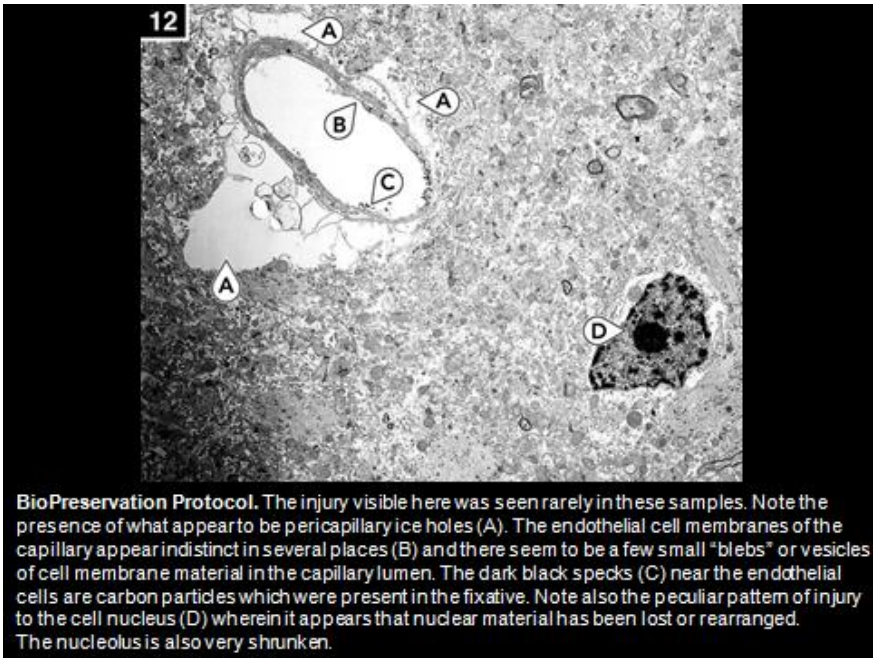
Indeed, we were able to demonstrate that even the finest resolvable structures, such as the synapses and their synaptic vesicles, were beautifully preserved and were, at least by electron microscopy, indistinguishable from comparable images of control tissue.



BioPreservation Protocol. The injury visible here was seen rarely in these samples. Note the presence of what appear to be pericapillary ice holes (A). The endothelial cell membranes of the capillary appear indistinct in several places (B) and there seem to be a few small "blebs" or vesicles of cell membrane material in the capillary lumen. The dark black specks (C) near the endothelial cells are carbon particles which were present in the fixative. Note also the peculiar pattern of injury to the cell nucleus (D) wherein it appears that nuclear material has been lost or rearranged. The nucleolus is also very shrunken.

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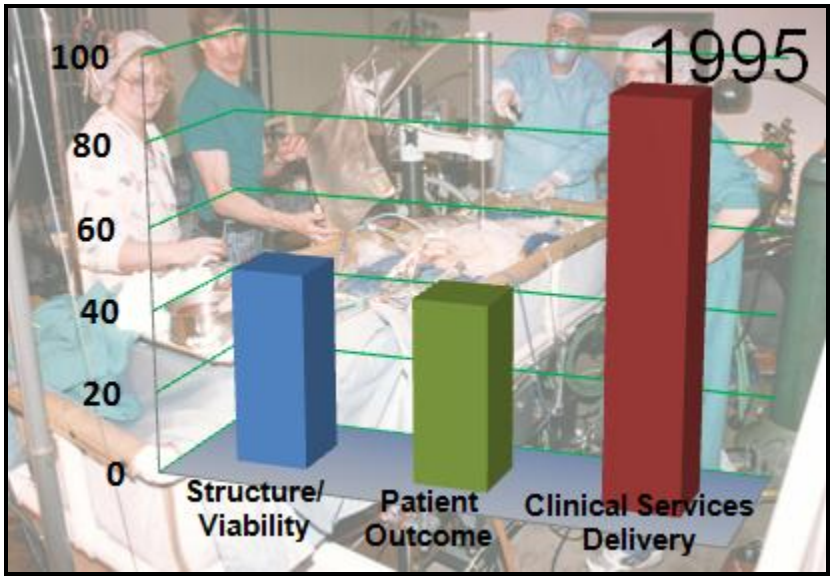
Notwithstanding the good overall appearance of brain ultrastructure using this protocol, there was still damage evident as a result of ice formation. Furthermore, such a high concentration of glycerol delivered at 10^{o}C (a necessity given its high viscosity) would result in the loss of cellular viability as a result of biochemical perturbation of proteins and lipids. Ultrastructural preservation was being achieved at the expense of molecular damage that would result in loss of viability.

I have taken this detour to emphasize the importance of research to the development of cryonics, and what's more, to demonstrate the importance of the hand-in-glove involvement in such research of the people who are delivering clinical care. It is my unshakeable opinion that separation of clinicians from researchers is untenable in

cryonics now, and at any time in the foreseeable time future.



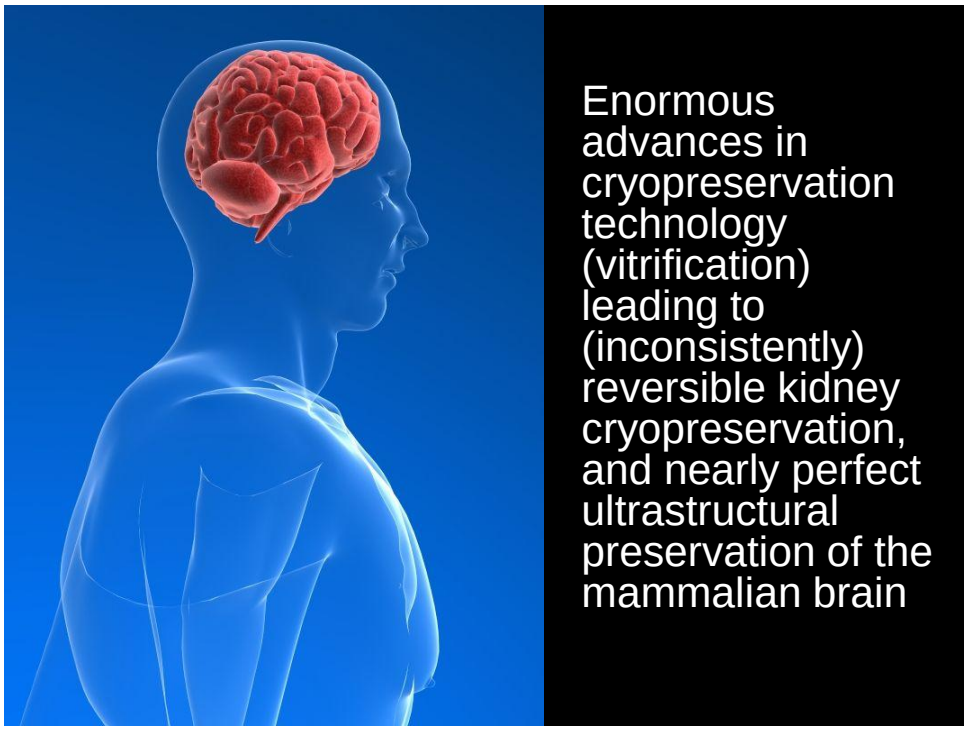
SLIDE 184

By1995 closed chest CPS had been further dramatically improved, pharamacoprotection against ~16 min of global normothermic ischemia in dogs had been demonstrated, and better cryoprotection in the form of 7.5 M glycerol had been adopted clinically. In effect what this meant was that *the only limitation on a patient remaining in a viable state during his interval of cryopreservation was the injury he incurred during loading with cryoprotectants and cooling to long-term storage temperature.*

While there was vast room for improvement in terms of logistics and prognostication vis a vis Clinical Services Delivery, the core technological requirements had been

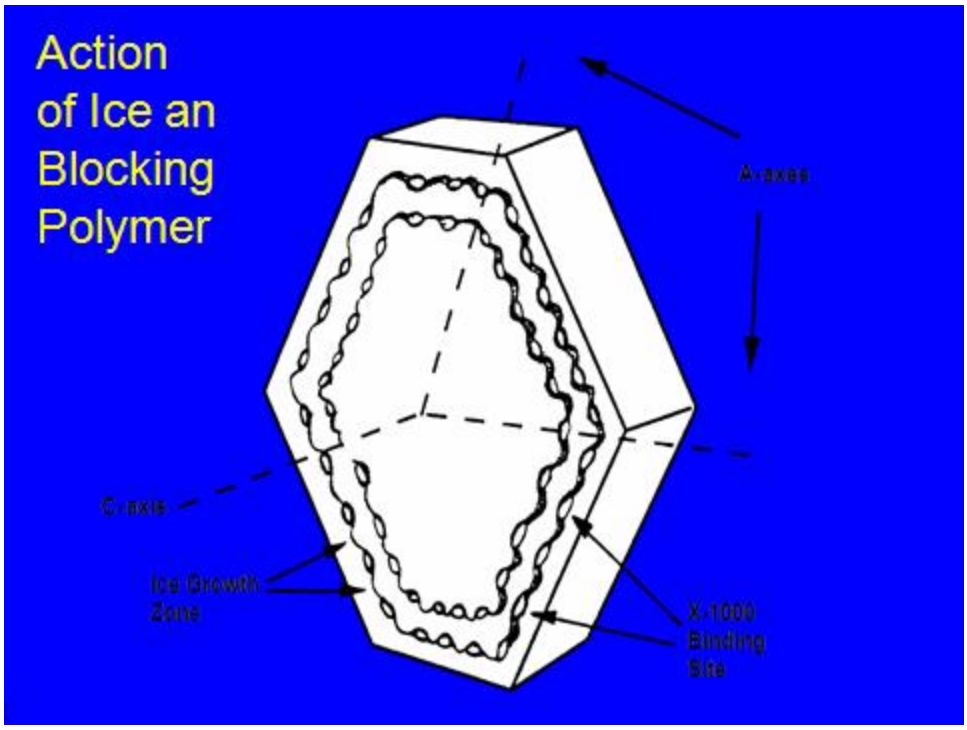
satisfied for any patient arresting under conditions that allowed for effective deployment of a Standby Team.

Whilst it would be highly desirable to extend the tolerable duration of asanguineous cold perfusion or cold storage (absent continuous perfusion) following blood washout to accommodate patients arresting remote from cryoprotection facilities, the existing technological platform employing the MHP-2 preservation solution developed by Cryovita in the early 1980s was adequate for patients in close proximity to CPA perfusion facilities. And of course, improved moderation of IRI injury would also remain a priority, particularly with respect to achieving sustained and high quality reperfusion in patients experiencing 17-60 min of normothermic ischemia. Nevertheless, by 1995 the most critical problem to be overcome was the injury resulting from the cryopreservation procedure itself.



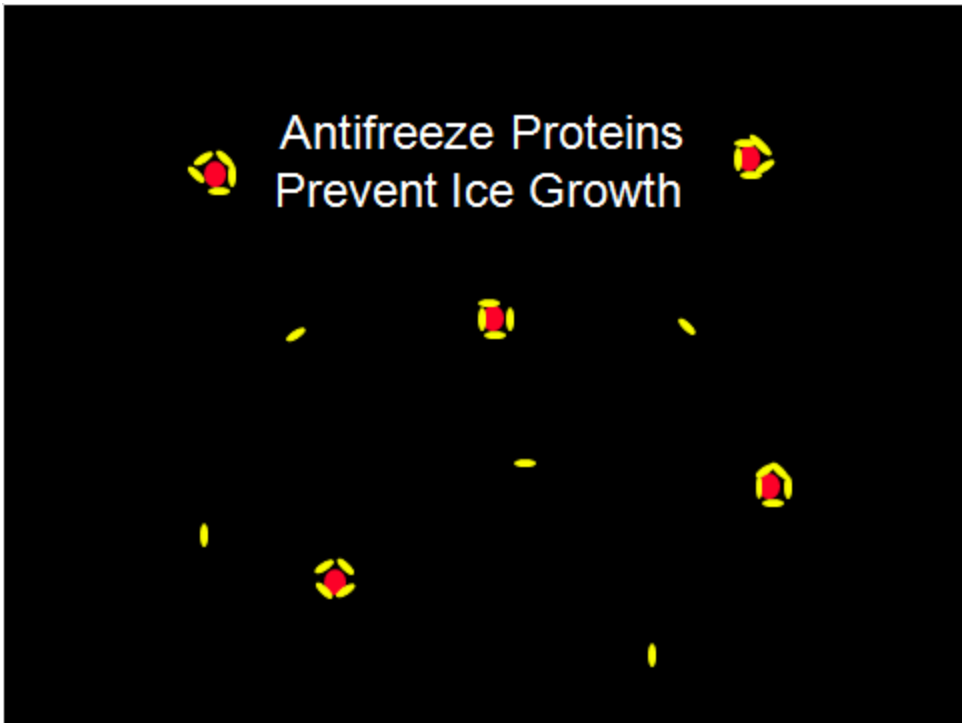
SLIDE 185

And that is where research efforts became focused. The US Red Cross was shuttering its organ cryopreservation program in Bethesda, Maryland at about the same time as Brian Wowk was completing his doctoral work in Medical Physics. Saul Kent acted aggressively to recruit both of these scientists in the closing years of the last century to work on the problem of reversible organ cryopreservation with an emphasis on the mammalian kidney and brain.



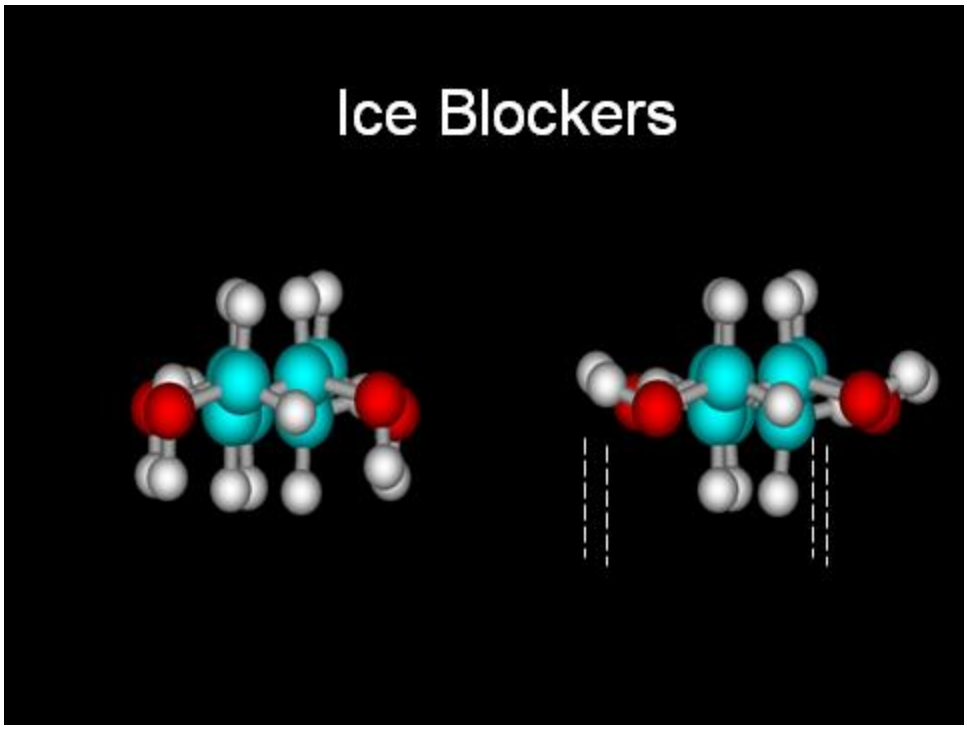
SLIDE 186

This work focused primarily on two lines of endeavor: increasing the stability and decreasing the toxicity of conventional colligative cryoprotectant solutions and developing novel polymers to inhibit ice growth in these improved solutions during cooling and rewarming; the development of so-called ‘ice blocking molecules.’ The latter was achieved in large measure by designing specific molecules to bind to the A and X axes of ice crystals, thus inhibiting or slowing their growth.



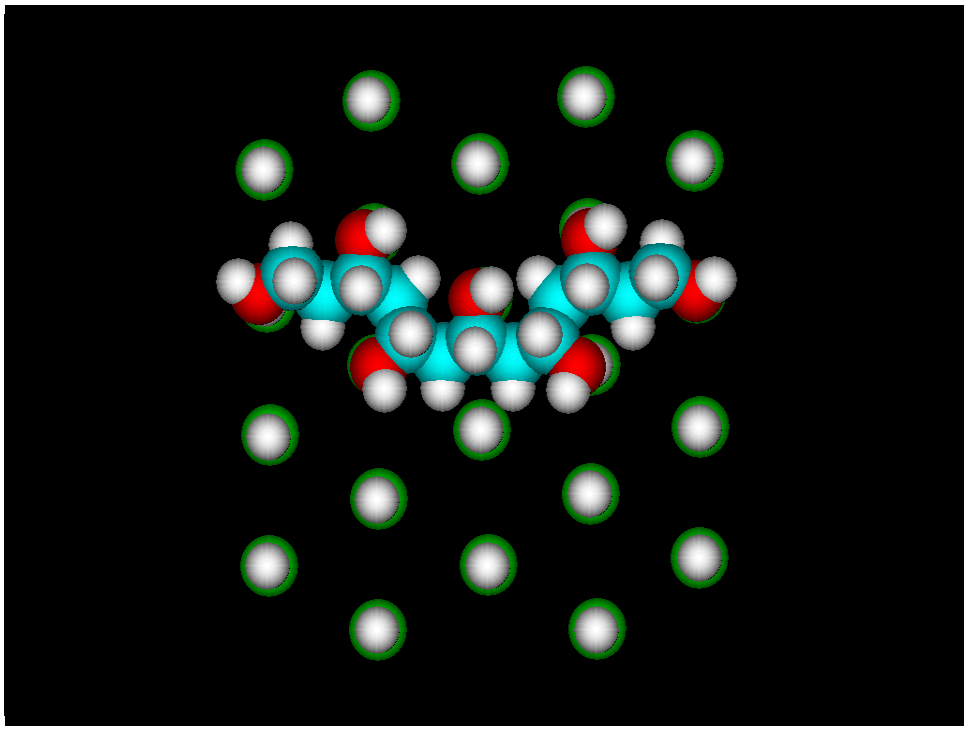
SLIDE 187

By developing synthetic versions of natural antifreeze proteins, Brian Wowk has made it possible to vitrify large volumes of cryoprotectant solution – or human tissue – eliminating all of the injury associated with freezing.



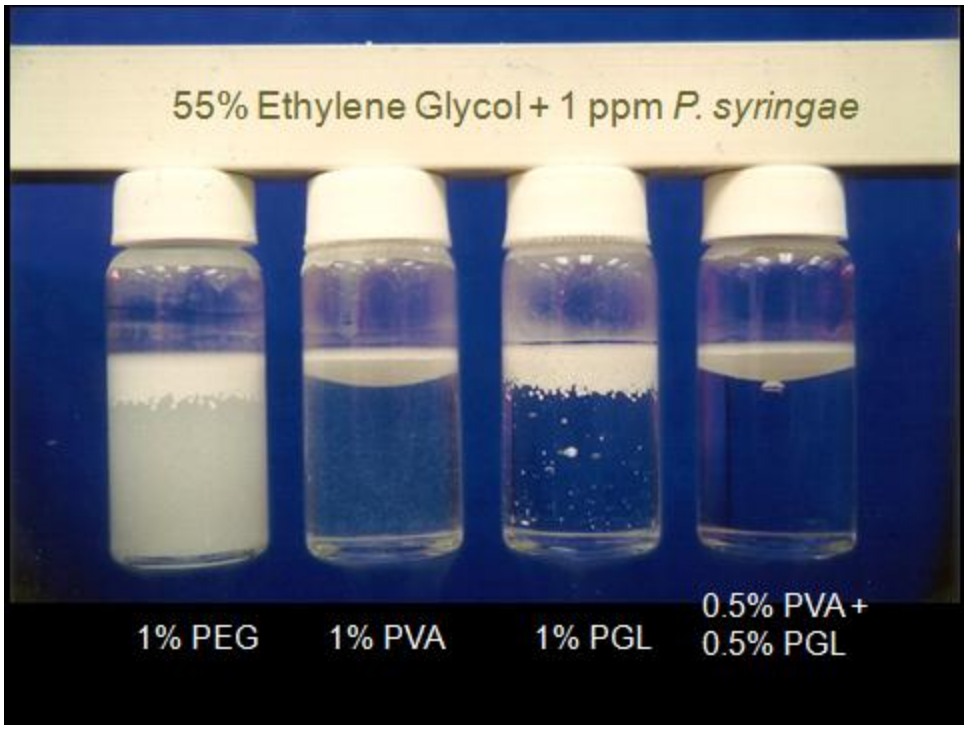
SLIDE 188

These synthetic molecules bind to the growing surface of ice crystals…



SLIDE 189

…and effectively halt their growth.

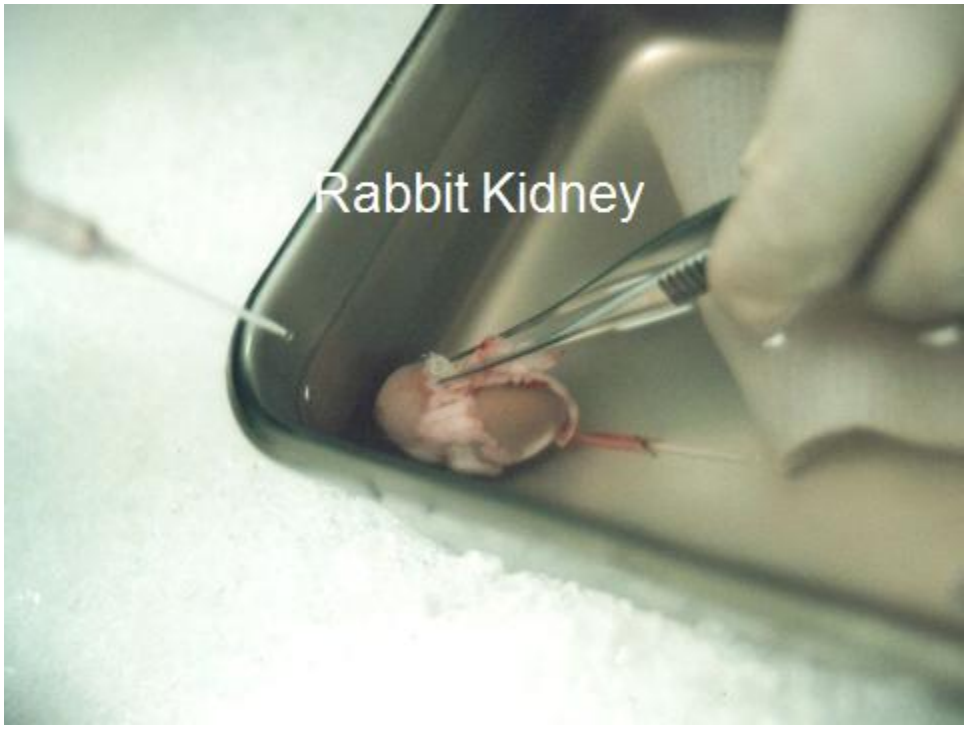


SLIDE 190

These compounds are stable and very inexpensive compared to the (protein) ice blockers found in nature, and they are vastly more effective and operate over a wider range of temperatures.

The powerful stabilizing effect of these molecules on cryoprotectant solutions undergoing slow cooling is evident here. As you can see in this slide, replacing just 1% of the solution with 0.5% of each of these two molecules results in the complete suppression of ice formation, even at very slow rates of cooling and rewarming; rates slow enough to allow for large mammalian organs such as the human kidney to be cooled to and re-warmed from stable vitrification storage temperatures in the range of ~ -145^{o}C without biologically damaging amounts of ice formation.

The maximum amount of ice tolerated by the rabbit kidney appears to be in the range of 2-3%. The best vitrification solution developed for the kidney to date is M22, whose critical cooling rate (the cooling rate above which ice formation is not observed) is 0.1°C/min, and whose critical warming rate (the warming rate above which ice formation is not observed) is 0.4°C/min.

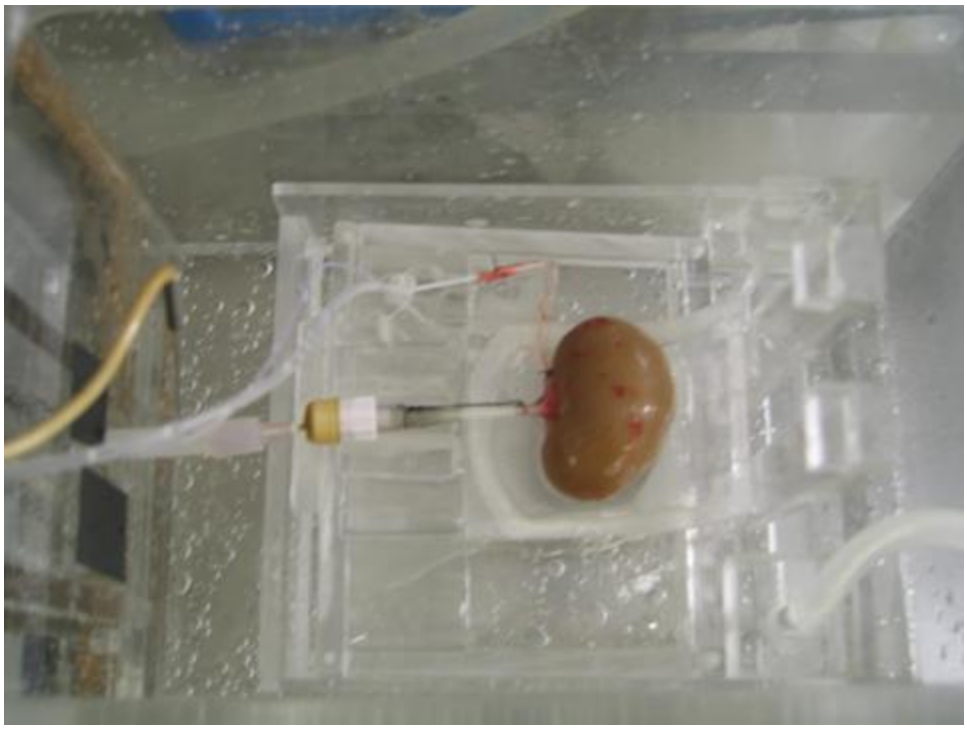


SLIDE 191

The use of one of these ice inhibiting molecules, X-1000, has been combined with a complex mixture of cryoprotectants present in sufficient concentration to allow for vitrification. This solution has been loaded into and unloaded from rabbit kidneys with consistent survival of the animals after re-implantation of the treated kidney and the immediate removal of the untreated one.

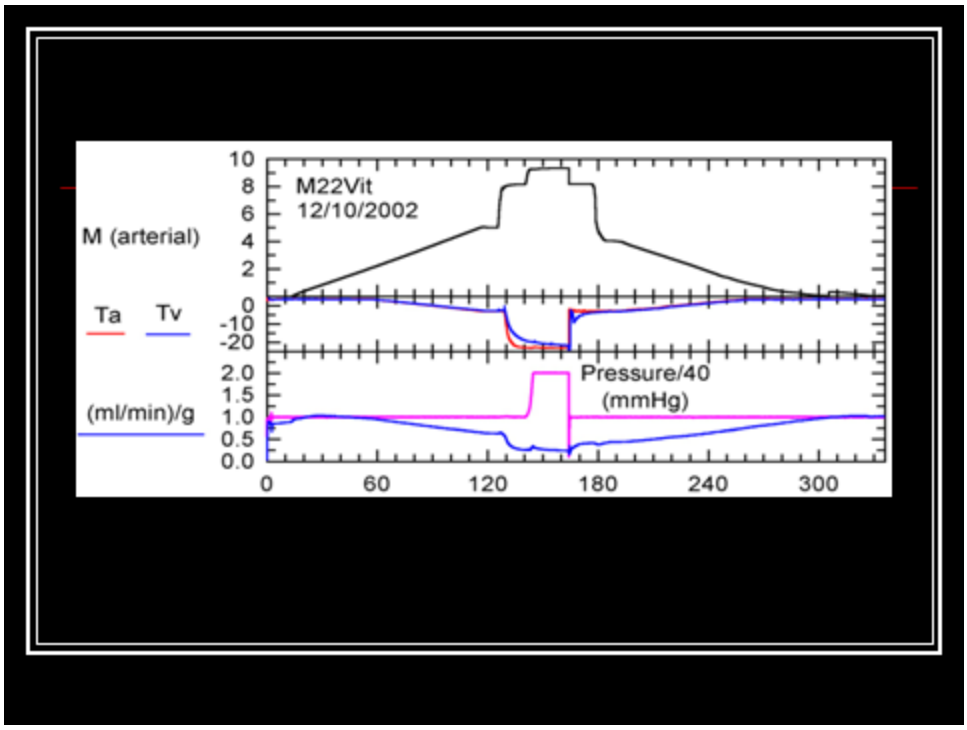
SLIDE 192

Sophisticated computer controlled equipment is required to carefully control the CPA introduction and removal process.



SLIDE 193

While the ice inhibiting molecules are not toxic in the concentrations needed, the other cryoprotectant agents are. As a consequence, perfusion to introduce these agents at their final concentration must take place at subzero temperatures.

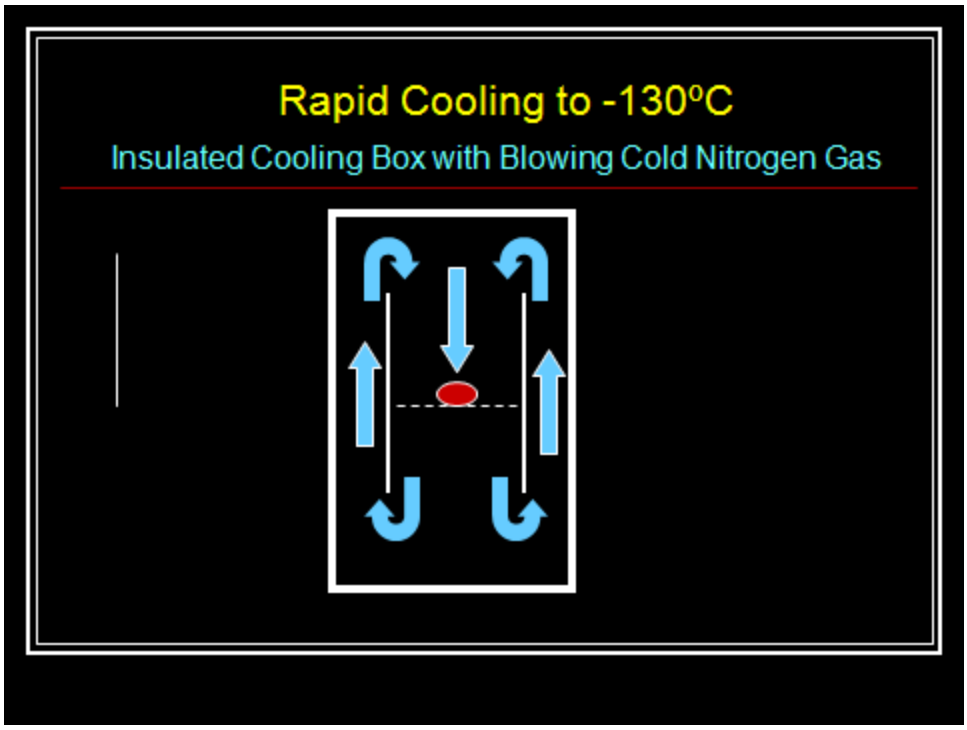


SLIDE194

This graph shows a typical plot of the CPA concentration (black), arterial temperature (red), venous temperature (blue) flow rate (blue) and perfusion pressure (pink) required to successfully load and unload a rabbit kidney with a vitrifiable amount of CPA. Note that the terminal temperature during CPA perfusion is -20^{o}C!

SLIDE 195

Kidneys cooled to and re-warmed from -140^{o}C show excellent cellular viability (~90%) and little macroscopic injury.

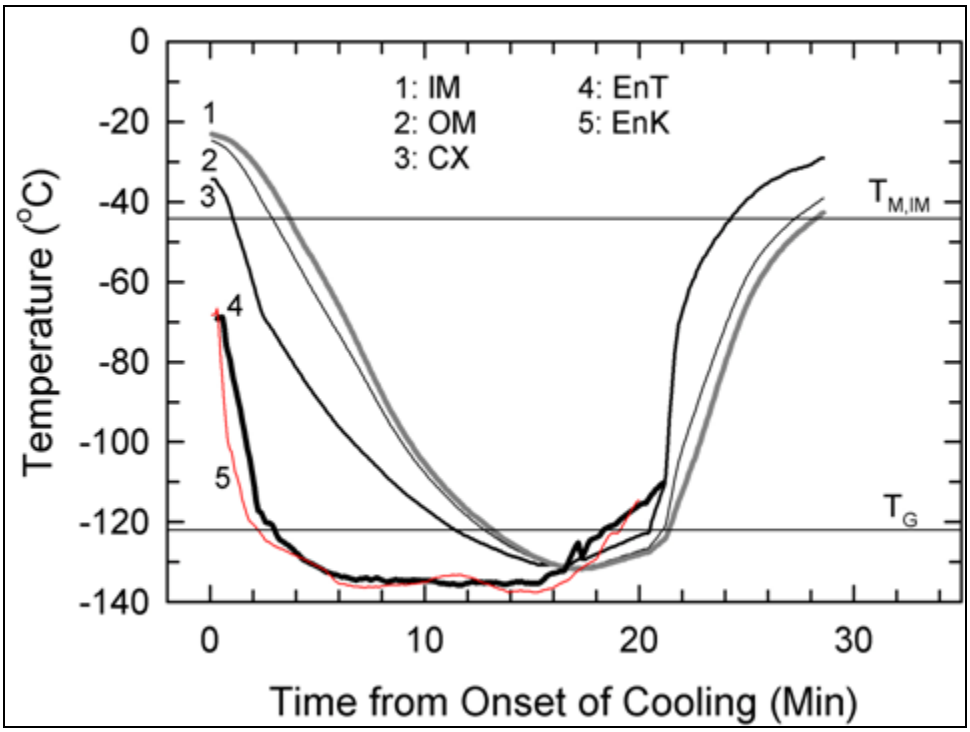


SLIDE 196

Rapid Cooling to -130°C is achieved by 'blast' cooling of the organ in an insulated cooling box using cold nitrogen gas circulated over the surface of the organ with a powerful squirrel cage blower.

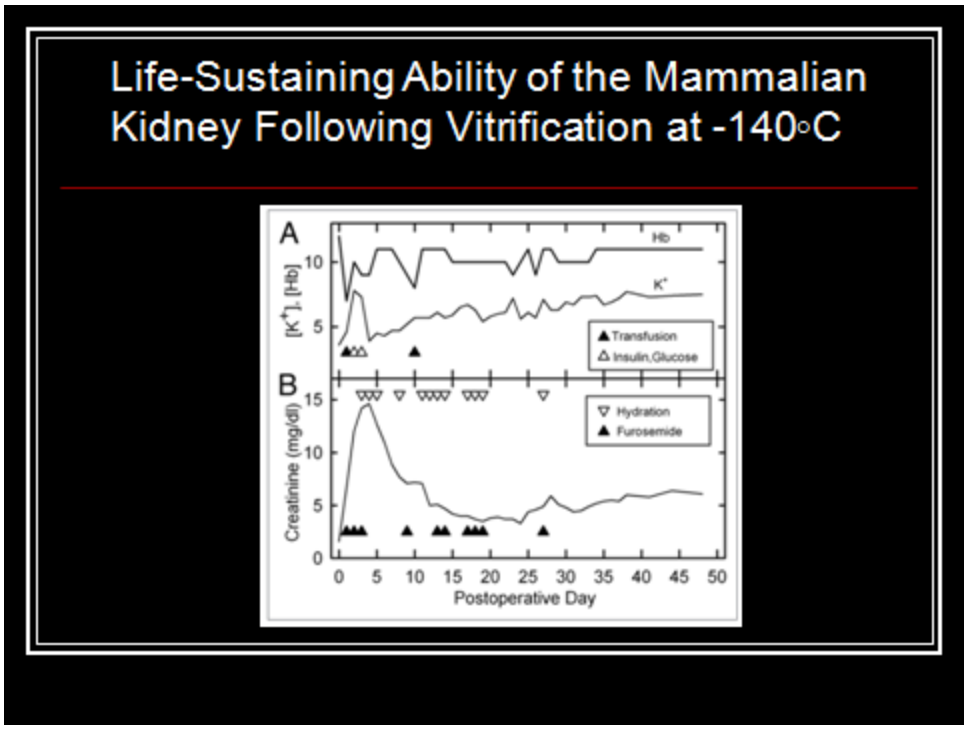
SLIDE 197

This kidney was cooled to and re-warmed from ~ -140^{o}C…



SLIDE 198

…as can be seen in this slide. What makes it special is that it supported the life of the animal as the sole kidney until the animal was sacrificed 48 days after the organ was re-implanted.

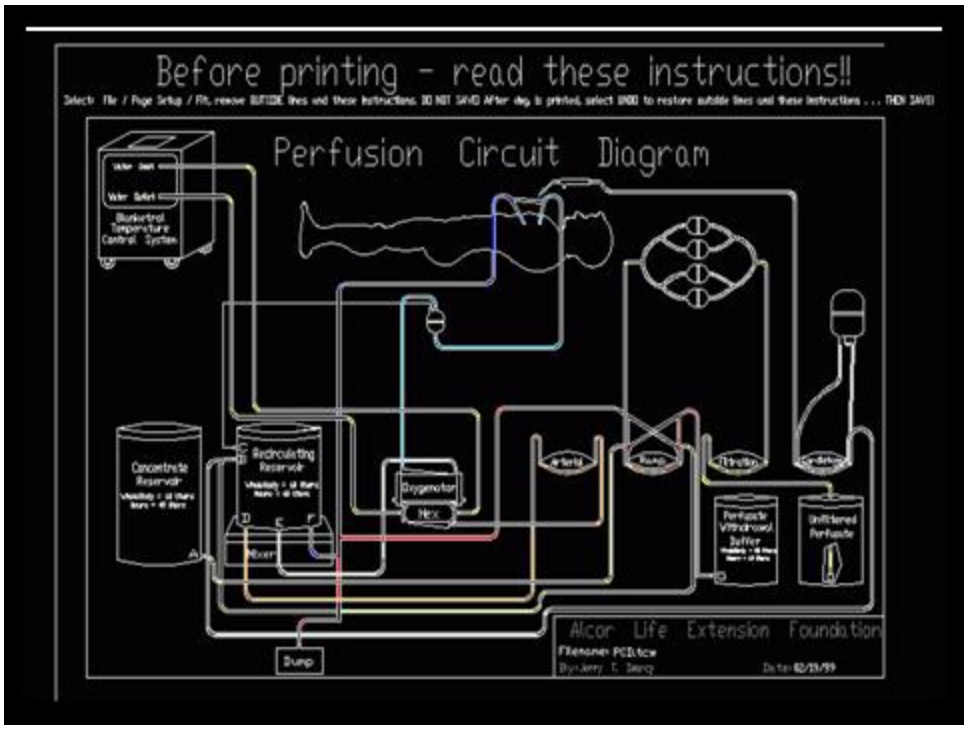


SLIDE 199

While far from perfect preservation, the survival of this single organ demonstrates an important 'proof of principle,' namely that a complex, highly vascularized mammalian organ, such as the rabbit kidney, can be cooled to and re-warmed from ~ -140^{o}C and still retain sufficient viability to support the animal. This is a major advance which will take on even greater significance if it can be replicated and improved upon.

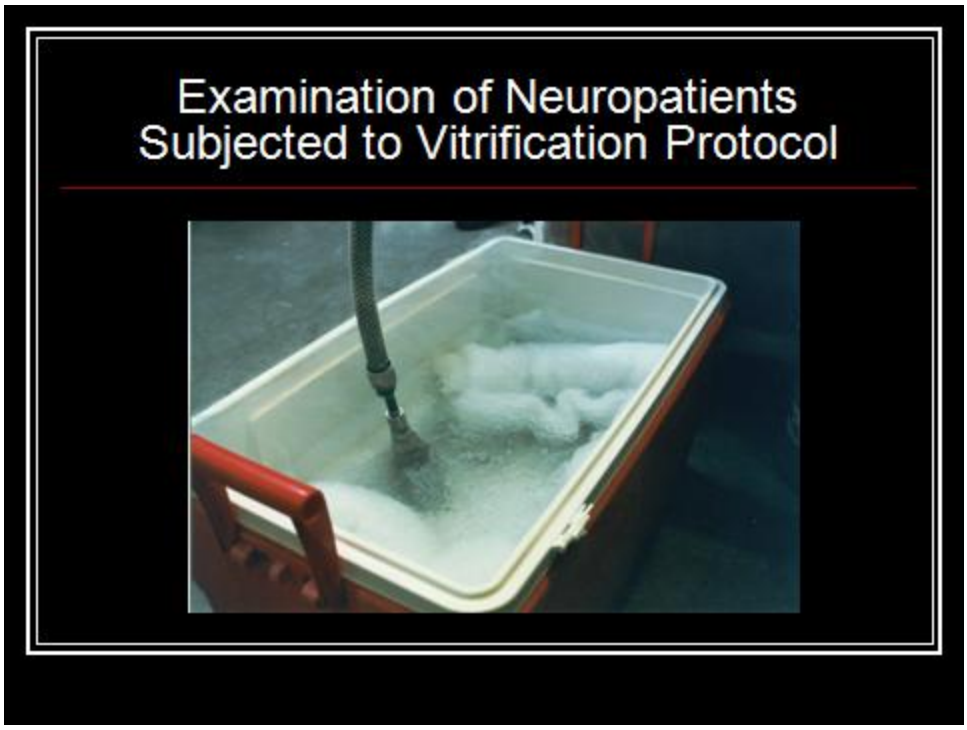
KEY TO GRAPH IN SLIDE: (A) Changes in blood levels of hemoglobin and potassium after transplantation of a previously vitrified rabbit kidney and interventions to correct both (triangles). Hyperkalemia was corrected by intravenous glucose (20 ml of 5% dextrose in 0.45% $NaCl^{-}$) and insulin (0.4 ml of 1 U/ml, IV). Anemia was corrected

with 20 ml of whole rabbit blood (~6–8 ml/kg) on each occurrence. Blood levels were measured before corrective interventions given on the same day. [Hb], hemoglobin concentration in g/dl); [K^+], potassium concentration in meq/l. (B) Postoperative creatinine levels and diuretic support history. Lower triangles indicate furosemide administration (generally 5–10 mg, IV or IM); upper triangles indicate hydration (generally 100–200 ml, consisting of equal volumes of 0.9% $NaCl^-$ and 0.45% $NaCl^-$ plus 5% glucose, subcutaneously). Blood levels were measured before corrective interventions given on the same day.



SLIDE 200

What have been the practical results of these efforts in terms of improved care for cryonics patients? The answer to that question is both complicated and frustrating. Both US cryonics organizations, Alcor and CI, claim to provide structural vitrification (i.e., not with conservation of viability) to their patients, and in the laboratory researchers at 21CM have demonstrated nearly complete vitrification of intact rabbits. So there can be little question that the capability to largely vitrify and to highly cryoprotect entire humans now exists in principle – in the laboratory.

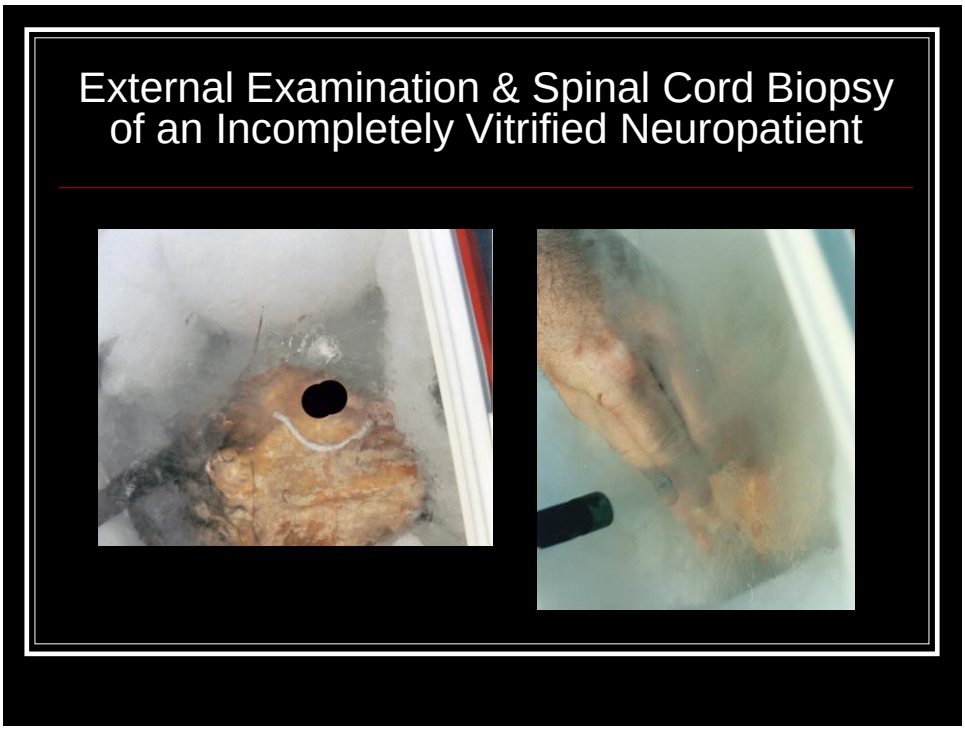


SLIDE 201

However, it is far from clear to what degree vitrification of the brain (leaving aside consideration of the rest of the body) is being achieved in practice. In 2002, with great difficulty and resistance, I persuaded Alcor to examine and photograph some of their neuropatients who had undergone their vitrification protocol. Quite incredibly (but given the history of cryonics, not unexpectedly) they had not been conducting *any* kind of examination on such patients and consequently they did not even know what the visual (external) appearance of a vitrified patient might be. By the way, the same was (and is) also true of the Cryonics Institute.

In the summer of 2002 three patients were removed from their long-term storage vessels and examined and

photographed while being continuously immersed in liquid nitrogen.



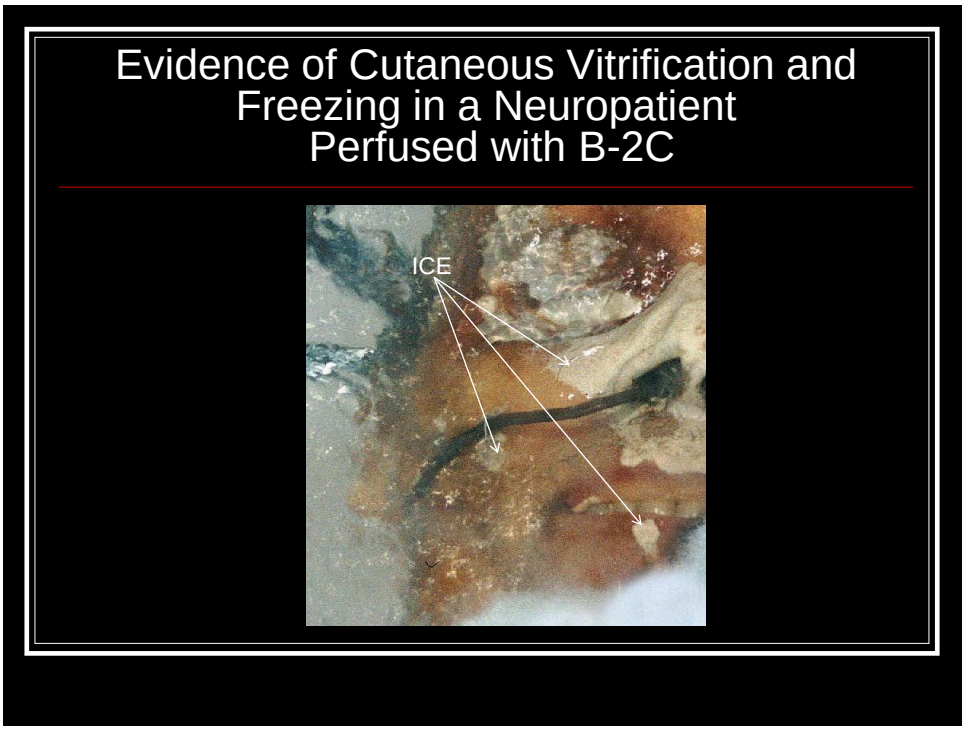
SLIDE 202

Not surprisingly, the examination of these patients returned a wealth of information. Superficial examination disclosed areas of the skin that were obviously vitrified, as well as areas that had clearly undergone significant freezing. In two of the three patients examined it was possible to visualize the cerebral cortical surface through the burr holes and see evidence of either freezing or vitrification. Note the extensive areas of freezing or partial freezing evident in this patient’s skin.

The image at the right documents that it was also possible to retrieve samples of spinal cord from the vertebral foramens of these patients.

SLIDE 203

Superficial examination of these cord samples showed no evidence of ice formation in one case, some evidence of freezing in the second case and what appeared to be frankly frozen cord tissue in the third case. The photograph at left is of apparently completely vitrified spinal cord while the photo at right shows what appear to be some islands of ice formation within the body of the cord.

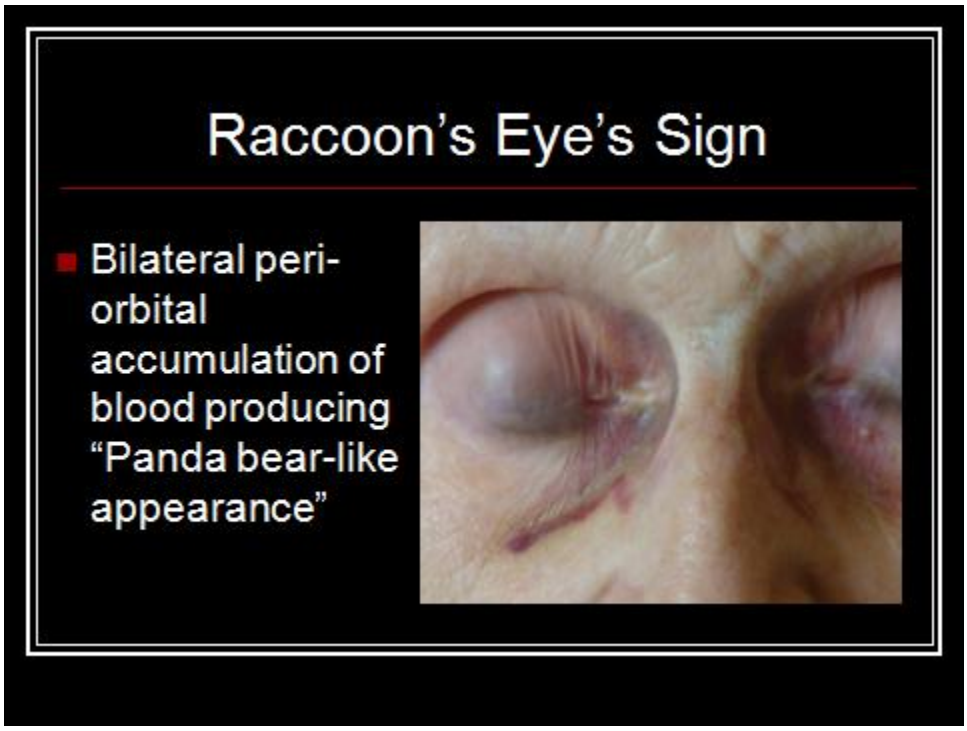


SLIDE 204

This image is of the neuropatient who demonstrated the most uniform cutaneous vitrification. The chalky white areas contain substantial ice, probably as a result of inadequate equilibration with the cryoprotectant solution. The small patch of freezing on the cheek adjacent to the temperature probe wire (entering the right nares) is the location of a skin staple used to secure the probe to the patient during Transport or cryoprotective perfusion. It seems likely that freezing occurred in the skin in this area due to compression of the tissue by the staple which interfered with the distribution of CPAs during perfusion.

However, the sharply demarcated freezing of the nares extending into the maxillary skin bilaterally and the

presence of bilateral periorbital ice in the skin were puzzling and seemed to have no explanation.



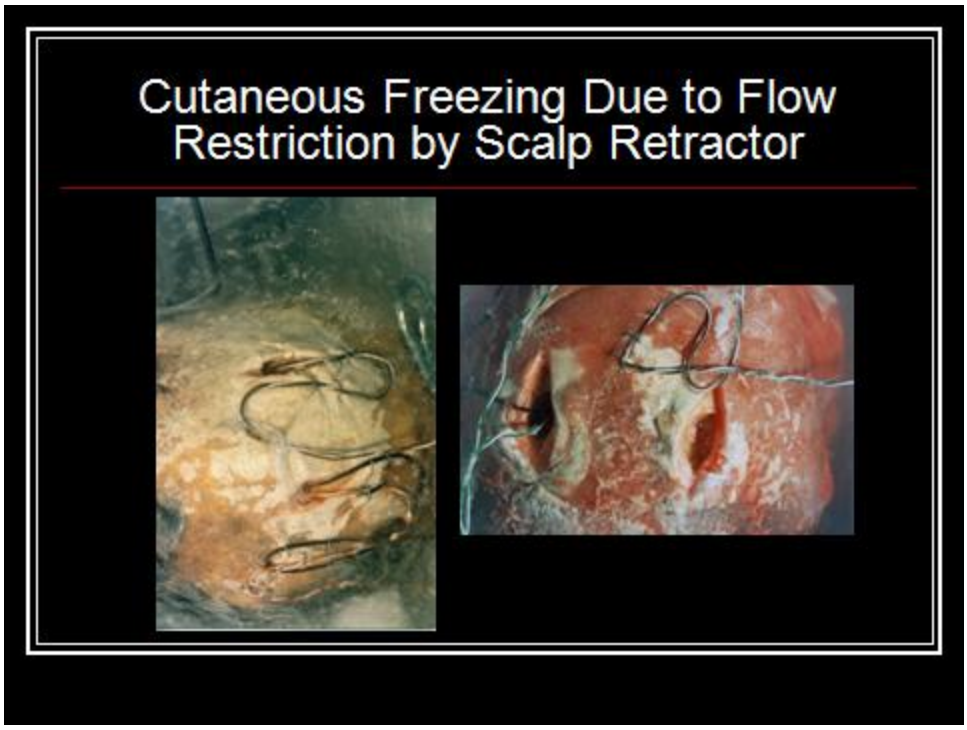
SLIDE 205

A careful review of this patient's hospice nursing notes disclosed that he developed 'raccoon's eyes sign' during his agonal decompensation. Raccoon's eyes sign, also called Panda eyes sign here in the UK, is most commonly associated with basilar fractures of the skull. It is also not infrequently seen in primary amyloidosis either as a spontaneous event, or after prolonged eye-strain. In layman's terms it is periorbital bruising as a result of the infiltration of blood into the soft tissues of the eye sockets.

Raccoon's eyes sign is also occasionally reported in patients with advanced cancer, and this patient was

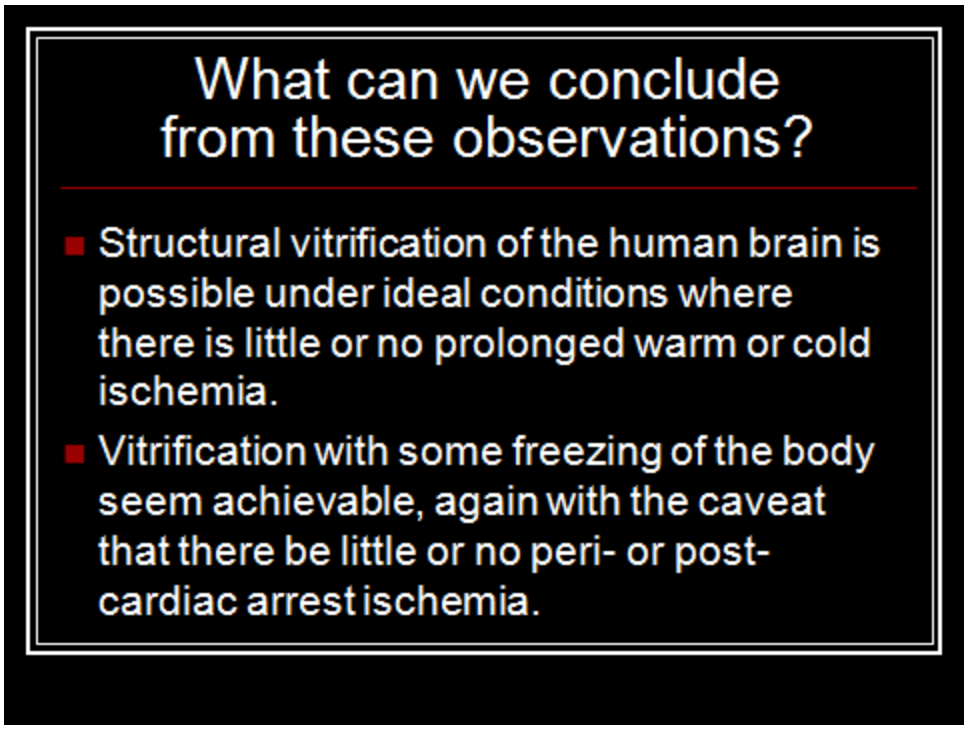
suffering from end-stage metastatic primary adenocarcinoma of the stomach. He had extensive carcinomatous invasion of his liver and may well have had coagulopathy secondary to liver failure or dysfunction. As was observed previously in dog and small animal work employing high molarity glycerol perfusion and freezing, any trauma to the skin and/or underlying tissues resulting in bruising also invariably compromises cryoprotectant equilibration. This phenomenon is seen in the tissues adjacent to surgical wounds for vascular access as well as in tissues adjacent to percutaneous devices such as arterial or IV catheters.

This patient proved impossible to intubate due to cervical anatomical anomalies secondary to long-standing ankylosing spondylitis, and as a result, an Esophageal Gastric Tube Airway (EGTA) device was used to facilitate ventilation. The mask of this device requires the application of a large amount of uninterrupted pressure in order to obtain an airtight seal for ventilation. This pressure, exerted for the prolonged period of time during which this patient was given external CPS, may have resulted in bruising injury to the tissues surrounding the nares thus compromising flow to the nose during subsequent cryoprotective perfusion.



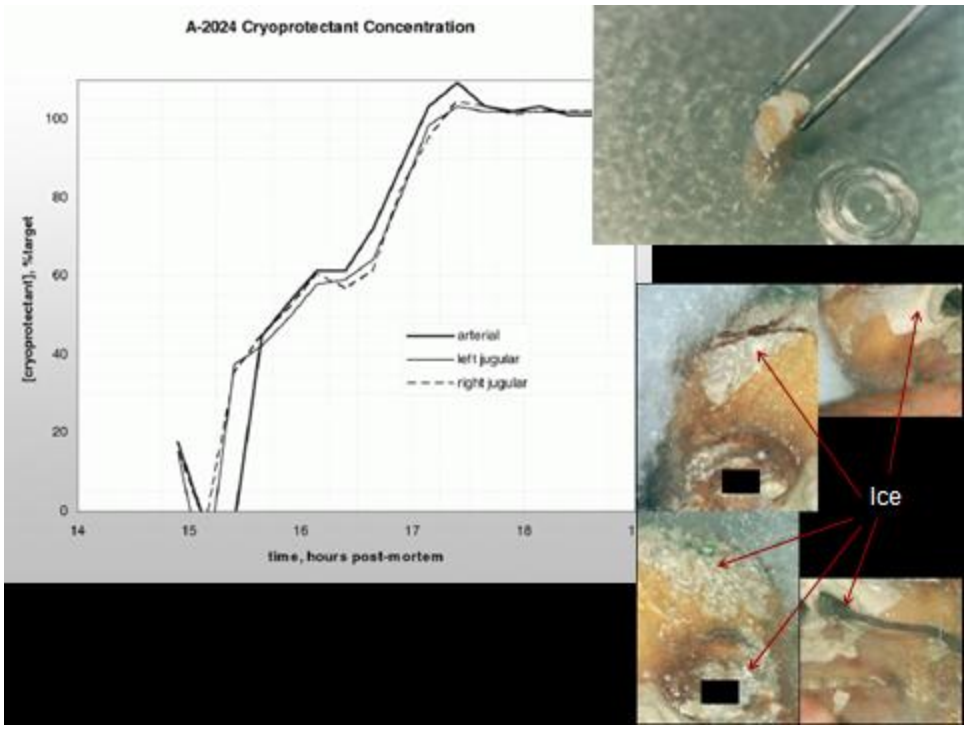
SLIDE 206

This phenomenon of failed vitrification was also consistently observed in the scalp adjacent to the burr holes of all the neuropatients that were examined. Again, this is almost certainly due to compression of the skin by the Weitlaner retractors that are necessary to retract the skin and allow visualization of the burr holes during CPA perfusion. Examination of the cut surface of the stump of the necks of these patients yielded similar results: evidence of vitrification as well as evidence of patchy freezing. Vitrification was most uniform in the patient who had received the best Transport followed by the most rapid post-arrest cryoprotective perfusion.



SLIDE 207

Human neuropatients have been loaded reasonably uniformly with vitrifiable concentrations of CPA and it seems likely that structural vitrification of the brain) and vitrification and partial freezing of rest of the body is possible under ideal conditions – conditions in which there is little or no warm and cold ischemia.



SLIDE 208

Examination of human cryopatients subjected to a vitrification protocol demonstrated to yield good ultrastructural results in animals has resulted in vitrification of those tissues visible to the eye, including the spinal cord, when the procedure is carried out on patients stabilized and transported under optimum conditions.

Unfortunately, it is not possible to speak to how well or poorly this protocol is actually preserving the ultrastructure of the patients to whom it is being applied and the reason this is so is instructive. The tissue samples collected from the ‘vitrified’ neuropatients at Alcor were never processed for light or electron microscopy as originally planned. After countless enquiries (and after making arrangements for the processing of these samples at

no cost) I have given up trying to find out what became of them. For the record, I have never received an answer of any kind: so far all I know they were processed and examined – or lost – or remain in storage.

As a result of the adverse publicity surrounding the Ted Williams case I have been informed that Alcor now prohibits photography of its patients before, during or after CPA perfusion. CI similarly prohibits photography and as of 2006, when last I checked, neither organization conducts any kind of examination of its patients after cooling to liquid nitrogen temperature. The working presumption is that "patients are vitrifying and if they are not, then there is nothing at this time that can be done about it."

A serious, and as far as I know unanswered question, is what happens to the ultrastructure of tissue that undergoes freezing at deep subzero temperatures – presumably without the benefit of slow extracellular ice formation resulting in cellular dehydration and ultimately cellular vitrification? Theoretically, cells freezing due to failed vitrification should freeze intracellularly due to supercooling and the absence of extracellular ice to translocate intracellular water and concentrate the CPAs inside the cells. Intracellular freezing results in vastly more damage than does conventional extracellular freezing with resultant vitrification of the intracellular milieu. This question takes on added urgency when considered in the context of the long warm and cold ischemic insults most cryonics patients currently experience. As we've previously seen, this has a profoundly deleterious effect on

ultrastructure in animals subjected to freezing with 4 M glycerol.

SLIDE 209

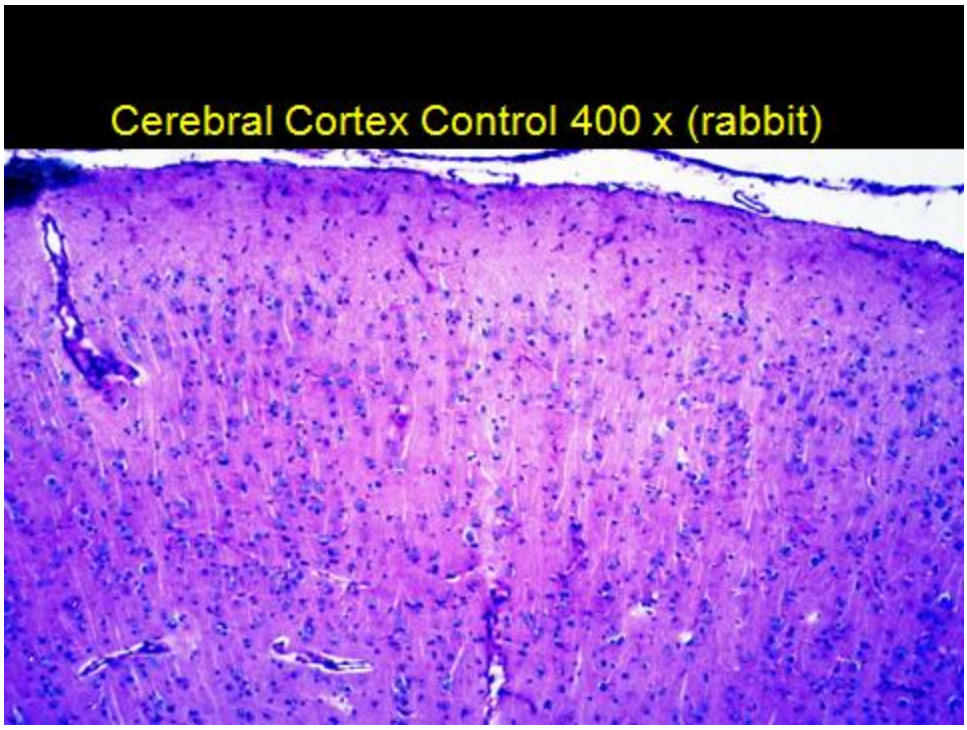
Finally, it is, as we have seen, quite easy to visualize significant amounts of ice formation in the skin and exposed (cut) tissues of human cryopatients subjected to vitrification protocols. The recent work of Fahy, et al., has demonstrated that the same is true for the interior of organs that have failed to vitrify completely. A principal reason for the failure to date to achieve reproducible and survivable vitrification of the rabbit kidney is that the medulla (the inner portion of the kidney where urine collection takes place) of the mammalian kidney is very poorly circulated and receives only a small fraction of the flow that the renal cortex receives. As you can see in the photo at the top there

is ice formation in the renal medulla during cooling to vitrification temperature.

Interestingly, while clearly visible to the eye, the amount of ice that has formed in the kidney in this slide constitutes only about 7% of the renal medullary mass! The maximum tolerable amount of ice formation appears to be in the range of 2-3%.

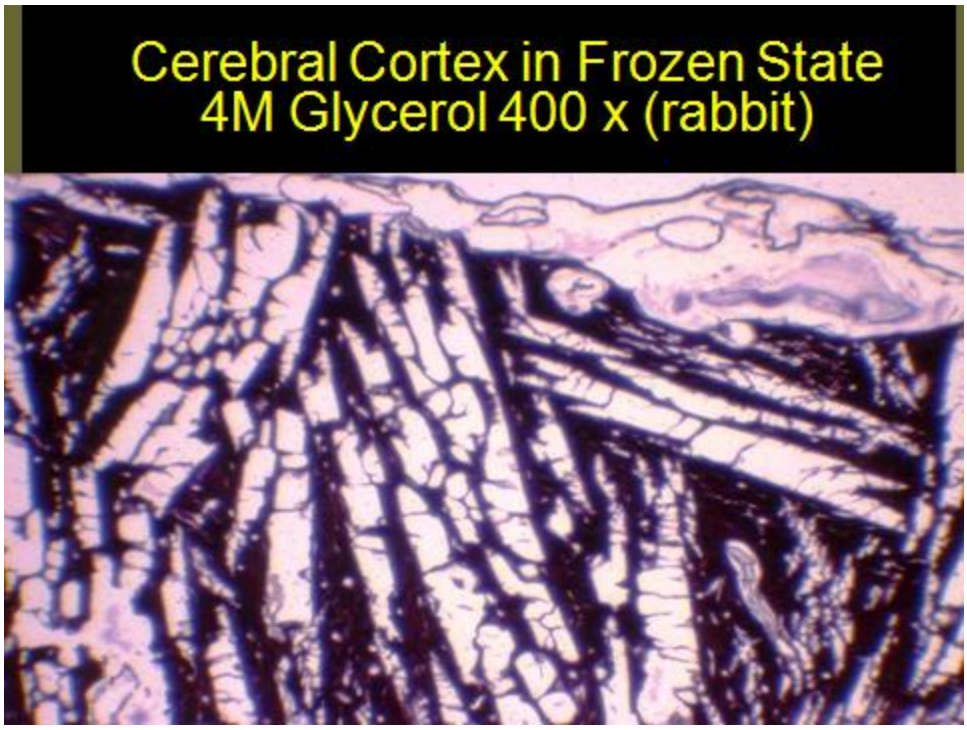
For obvious reasons it is not possible to image ice formation in human cryopatients by the expedient of sectioning them in half. But it *is* possible to better bound the problem by expanding the size of the burr holes to allow for better post-cooling examination of the cerebral cortical surface. The brain, much like the kidney, has a 'super' perfused cortex relative to its mostly low flow, myleinated interior. There also remains the possibility of imaging the brain and body of patients in some way that would allow for the visualization of areas that have failed vitrification. Radio frequency imaging of tissues for cancer detection is now emerging and perhaps this technique, or a variation, might be applicable to cryonics patients.

Regardless of whether such non-invasive imaging is possible or not, the failure of cryonics organizations to diligently explore and obtain feedback from a thorough visual inspection of their patients, and to document it photographically, is deeply troubling.



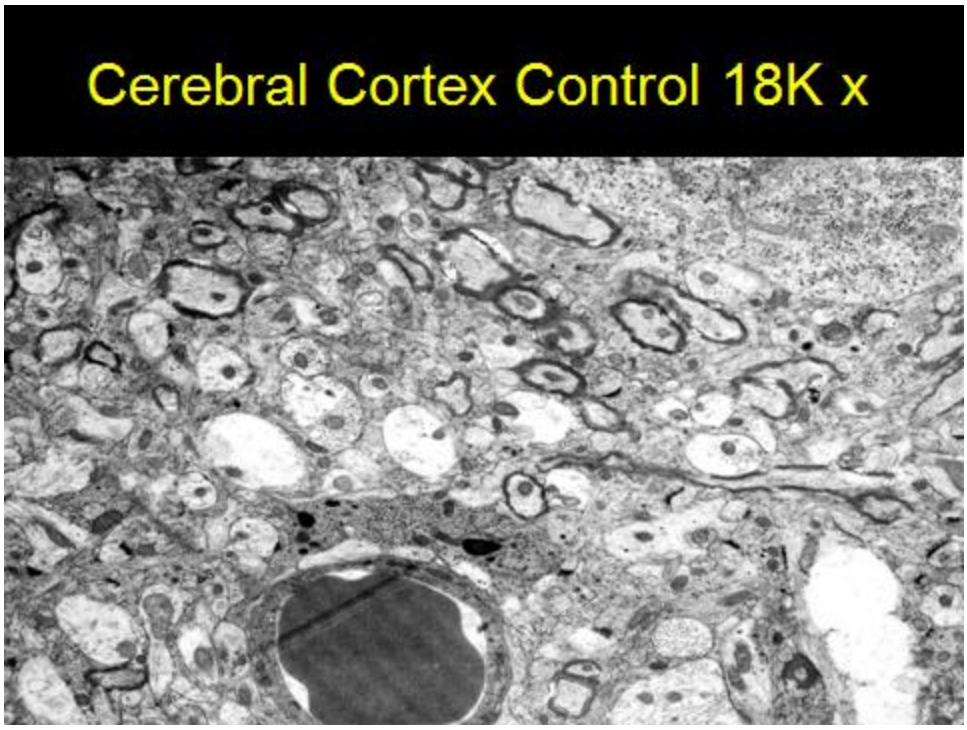
SLIDE 210

What are the implications of this progress in terms of potentially improved ultrastructural preservation of cryonics patients? What you see here is rabbit cerebral cortex stained and magnified 400 times. This is how healthy rabbit brain looks when prepared for examination under the light microscope.



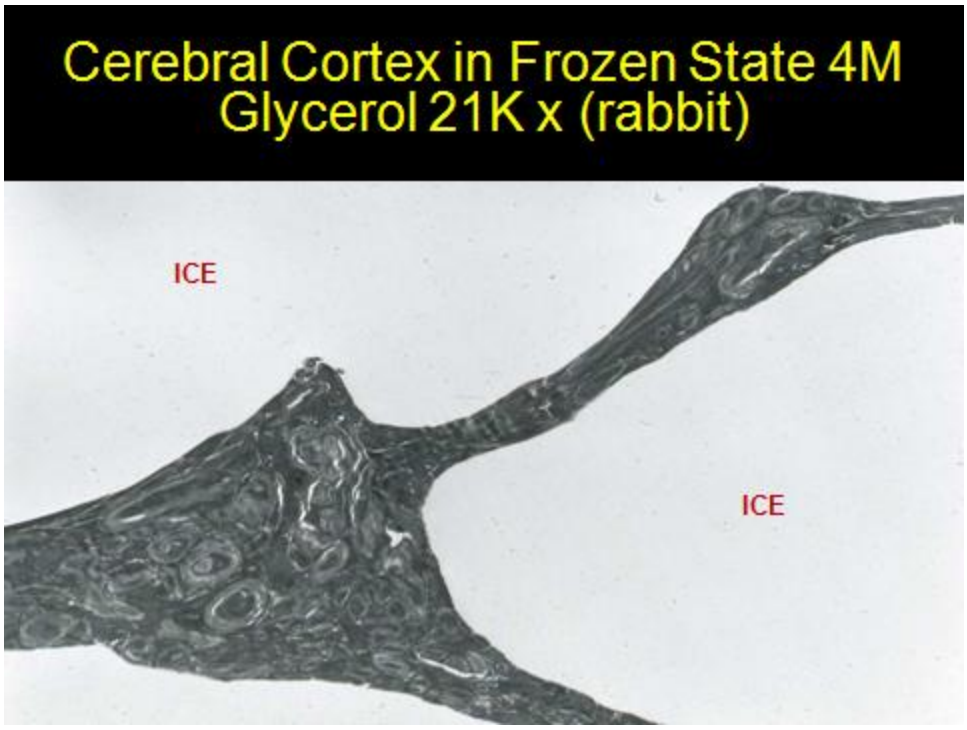
SLIDE 211

What you see here is rabbit brain in the frozen state. A technique called freeze-substitution is used to dissolve away the ice at -70^{o}C and the tissue is then fixed, stained and prepared for microscopy. Every white space you see here is ice! The damage from this ice formation, even in the presence of 4M glycerol, is enormous.



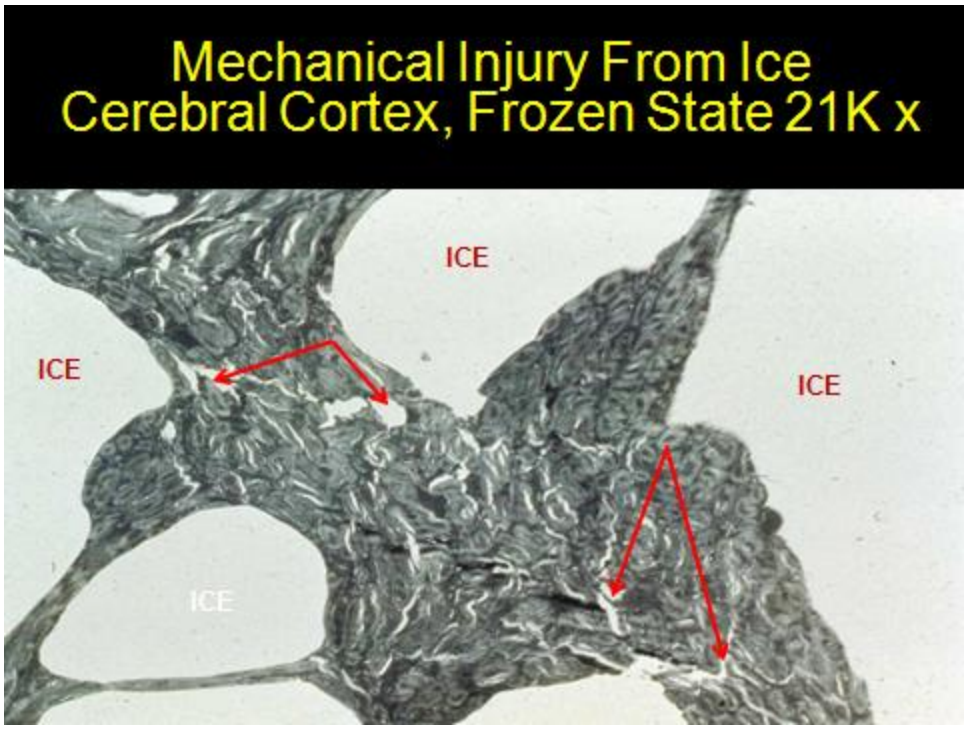
SLIDE 212

This is what normal rabbit cerebral cortex (hippocampus) looks like when prepared for electron microscopy from a healthy, living animal. This is our control or reference slide for looking at the next image, which is rabbit brain in the frozen state after perfusion and freezing in the presence of 4M glycerol.



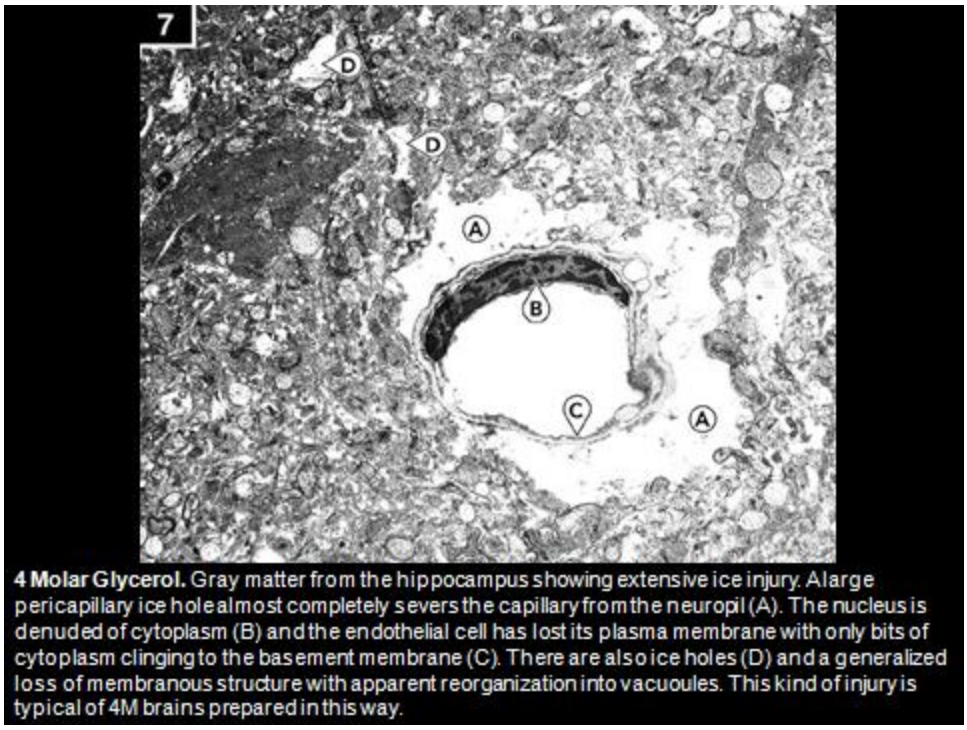
SLIDE 213

If we increase the magnification of frozen brain tissue to 21,000 times we can clearly see the brain parenchyma compressed between masses of ice.



SLIDE 214

And if we look closely, we can see tearing and disruption of cellular and intercellular structures as well as the presence of fractures.

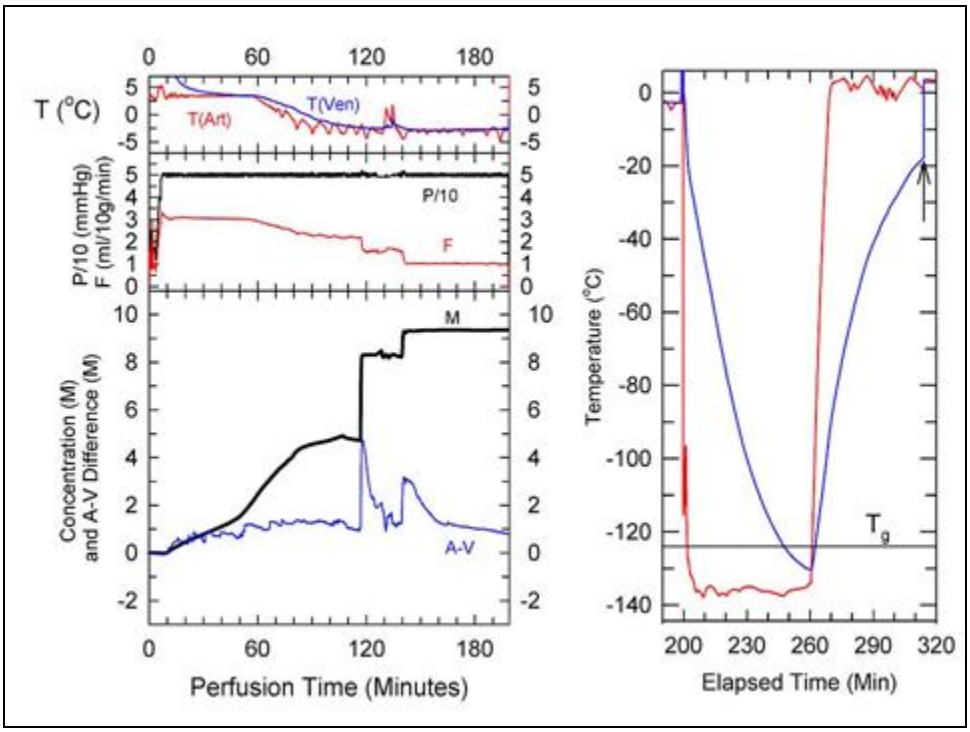


4 Molar Glycerol. Gray matter from the hippocampus showing extensive ice injury. A large pericapillary ice hole almost completely severs the capillary from the neuropil (A). The nucleus is denuded of cytoplasm (B) and the endothelial cell has lost its plasma membrane with only bits of cytoplasm clinging to the basement membrane (C). There are also ice holes (D) and a generalized loss of membranous structure with apparent reorganization into vacuoules. This kind of injury is typical of 4M brains prepared in this way.

SLIDE 215

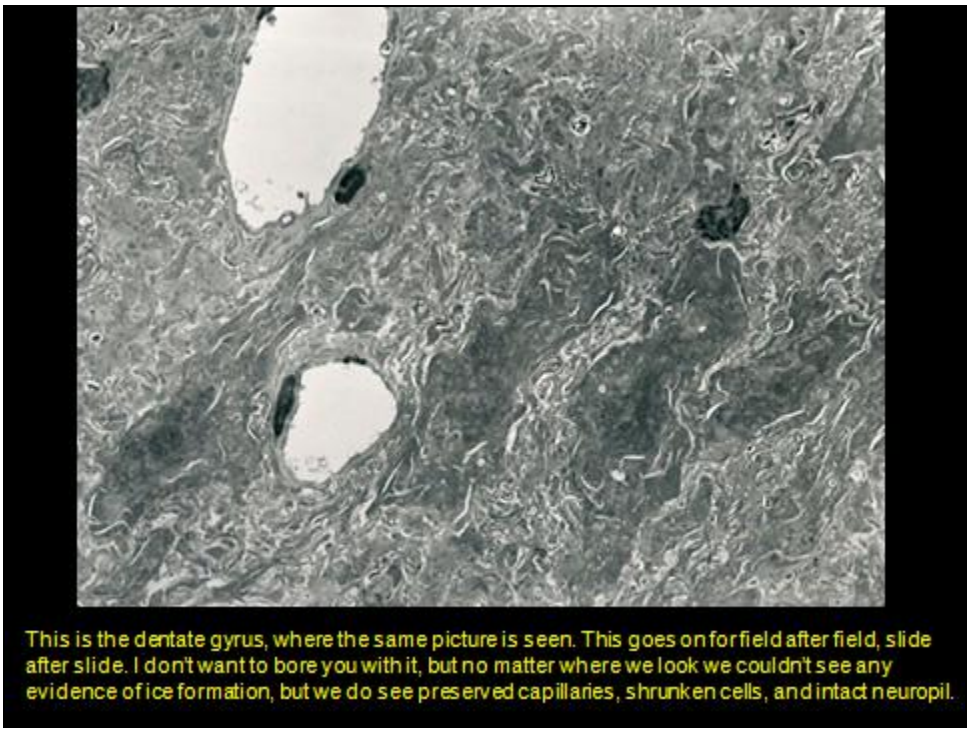
If we then thaw the system out and examine it; we see the full extent of the damage. 4 M Glycerol. Gray matter from the hippocampus showing extensive ice injury. A large pericapillary ice hole almost completely severs the capillary from the neuropil (A). The nucleus is denuded of cytoplasm (B) and the endothelial cell has lost its plasma membrane with only bits of cytoplasm clinging to the basement membrane (C). There are also ice holes (D) and a generalized loss of membraneous structure with apparent reorganization into vacuoles.

This kind of injury is typical of 4 M brains prepared in this way.



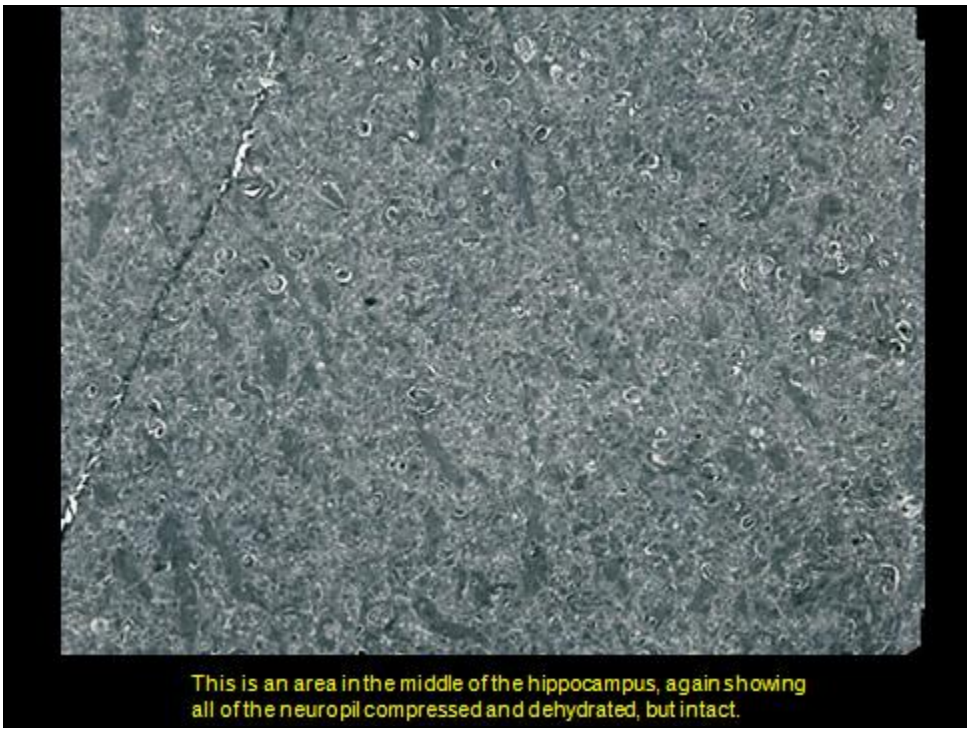
SLIDE 216

By contrast, if prepare rabbit brains using the M-22 protocol illustrated here…



SLIDE 217

…we see no ice formation whatsoever and thus no mechanical injury to the tissue on either the histological or the cellular levels.



SLIDE 218

The architecture of the neuropil – the fine connections between neurons – appears completely intact. It is not possible to achieve the same degree of fine structural resolution using electron microscopy with these tissues because they are intensely dehydrated from the vitrification solution – and in fact, this dehydration causes mechanical injury to the interface between the brain capillaries and the brain parenchyma. We did not realize that these peri-capillary holes, which are also seen in glycerolized frozen brains, were due to dehydration injury of the brain from cryoprotectant until it became possible to vitrify brains. Prior to being able to vitrify brains it was thought that they were artifacts of ice formation.

REVERSIBLE BRAIN CRYOPRESERVATION?

SLIDE 219

While there is significant dehydration from the CPAs, the overall structure of the tissue is intact; cell-to-cell connections appear perfectly preserved and intracellular structures are in place and free of mechanical disruption.

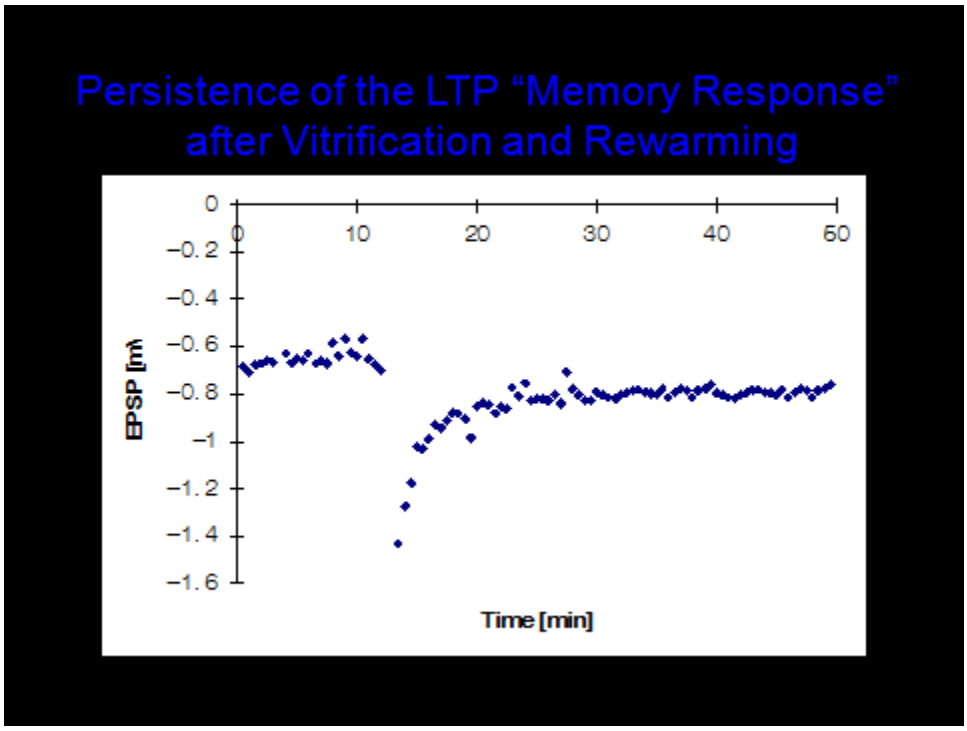
We know this because upon rewarming and evaluation for viability, brain slices subjected to vitrification exhibit a return or normal electrical activity, and the connectivity of various neuronal 'circuits' can be demonstrated by applying electrical stimulation and measuring the resulting evoked potential in the brain slice EEG. This work was carried out by 21CM several years ago, but to my knowledge, has not been published.

SLIDE 220

In April of 2006 21CM published data documenting the survival of vitrified mammalian brain slices. While this is encouraging, it is still a long way from achieving viability in an intact mammalian brain. One reason for this is that it has not, so far, been possible to carry out deep subzero perfusion of brain as can be done with kidneys.

There is failure of flow at temperatures much below -4^{o}C. Since brain slices can be immersed in the perfusate they are to be equilibrated with there is no such limitation on the temperature at which the vitrification CPAs can be introduced. Until less toxic vitrification solutions are developed, or a way is found to introduce existing solutions at lower temperatures to circumvent their toxicity, the goal

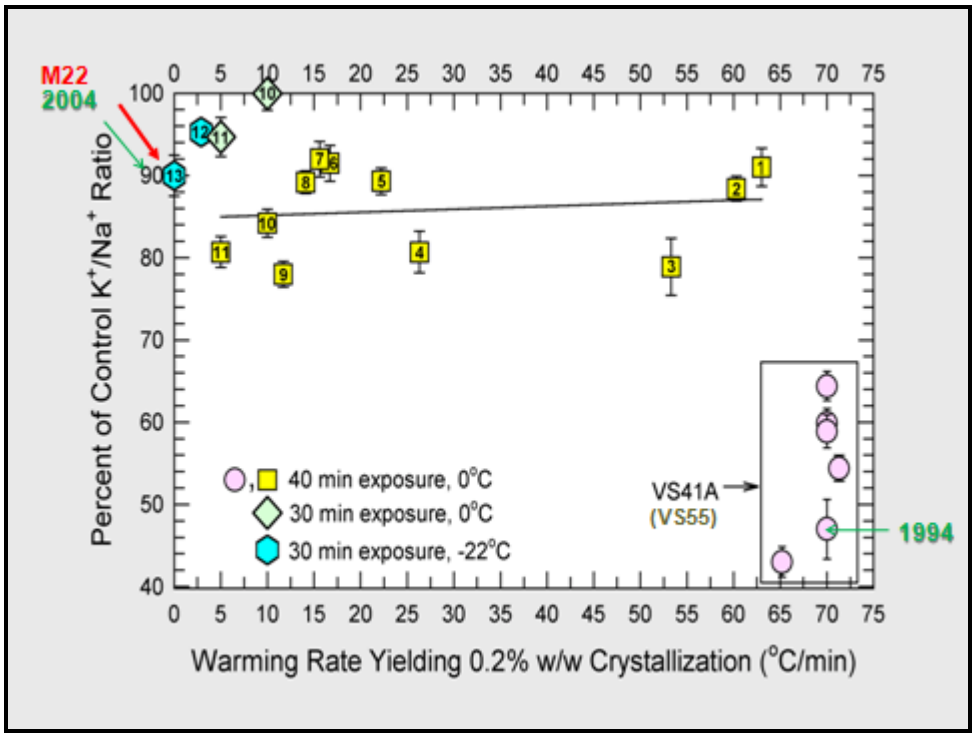
of reversible cryopreservation of the intact brain will remain out of reach.



SLIDE 221

Perhaps most impressively, 21CM has recently demonstrated that long term potentiation (LTP), the mechanism by which it is believed memory is initially encoded in the brain, survives cryopreservation by vitrification. LTP was robustly preserved in brain slices subjected to vitrification and subsequent re-warming and evaluation.

TAKING STOCK OF OUR PRESENT PERDICAMENT



SLIDE 222

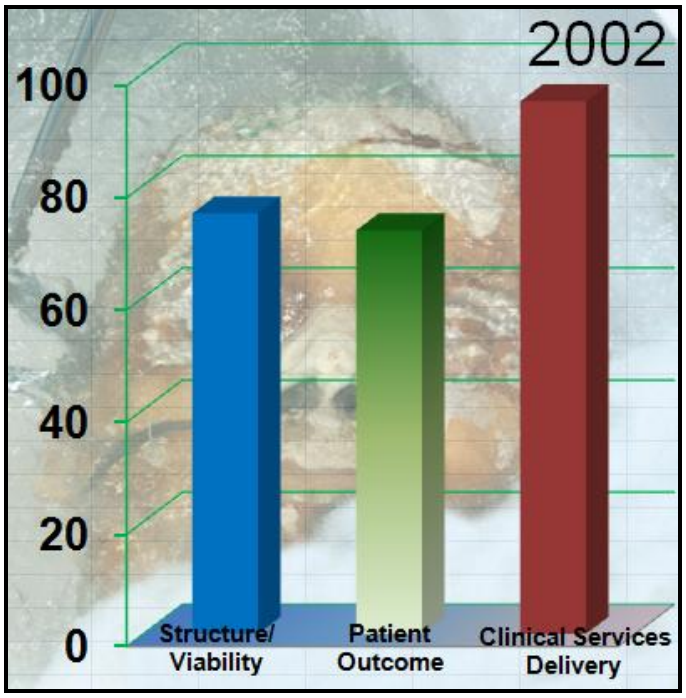
The work of Fahy and Wowk has brought us to the edge of reversible organ cryopreservation. It is no longer the case that cryopatients' brains need be macerated by ice as a result of freezing. Patients stabilized immediately post-arrest and perfused under good conditions can enter a state of nearly ice free cryopreservation with very little histological or ultrastructural disruption of the brain.

This slide documents the progress towards achieving viable cryopreservation of the kidney. It is worth saying a few words about this work if for no other reason than to

give some sense of the rate of progress: which has historically been quite slow.

This is what is called a ‘viability-stability diagram’ and it depicts the relationship between the stability of a solution to devitrification and its impact on tissue viability. The desirable place to find yourself on this graph, whether you are a kidney or a cryonics patient undergoing vitrification, is in the upper left corner.

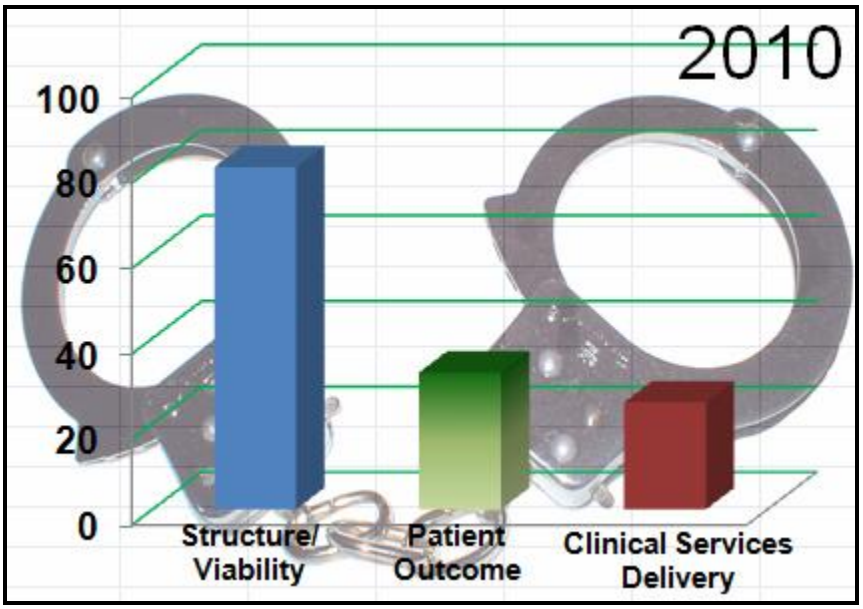
You want to have 100% viability and be completely stable – invulnerable to ice formation - even during cooling and rewarming at very slow rates. In 1984 VS41A was the best solution available and it yielded ~45% viability at a critical warming rate of ~70^{o}C/min! By contrast, in 2007, M-22 and a successor solution were yielding tissue viability between 90 and 95% at a critical rewarming rate of ~3^{o}C/min.



SLIDE 223

With these considerations in mind, let us briefly return to the performance graphs for cryonics that we began this discussion with. If the maximum score for currently available structural preservation is adjudged to be at ~75% - about as high as it can be absent viability, then what is the highest Patient Performance Score likely to have been achieved so far? The short answer to that question is that we don't know because the histological and ultrastructural evaluations on the candidate patient were never done. However, if we presume (from the patient's records) that his care was such that he experienced very little ischemic injury, then his score might be in the vicinity of ~73%.

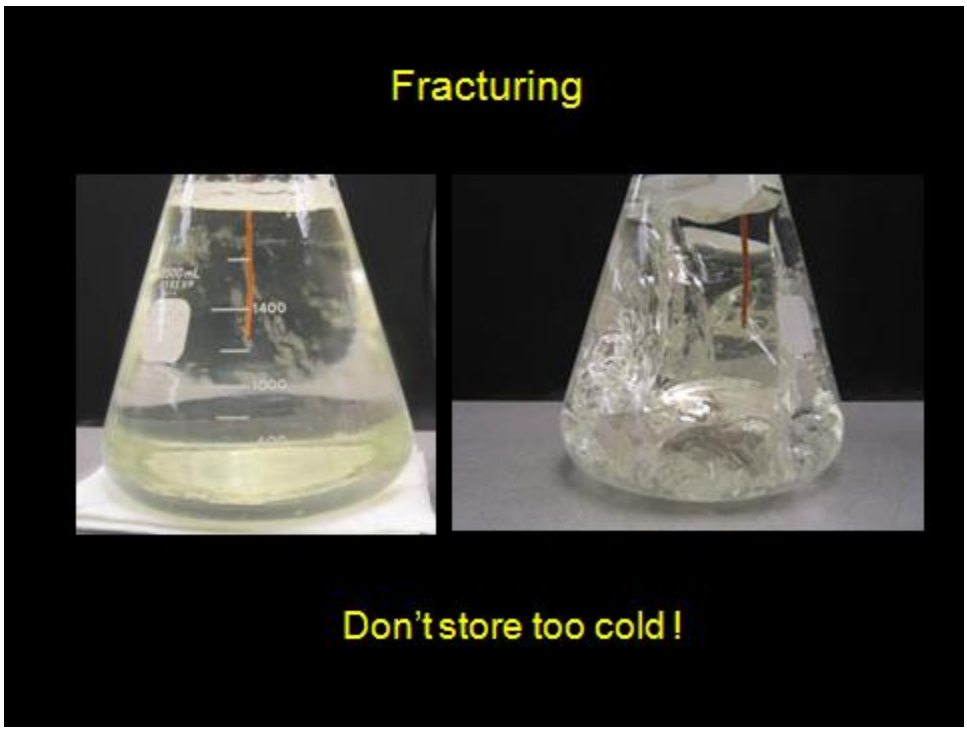
That is an impressive and unprecedented gain.



SLIDE 224

Unfortunately, if we also take a hard look at the quality of clinical care most cryonics patients are receiving it seems unlikely, bordering on impossible, that they are realizing much benefit from ‘vitrification’ as it is currently being practiced. The technology to deliver cryopreservation where the only injury is comparatively ‘trivial’ injury on the molecular level (denaturation of some proteins and rearrangement of the structure of some lipids) now exists, but is effectively handcuffed by the absence of a culture and a profession capable of delivering it.

ATTENUATING FRACTURING



SLIDE 225

Finally, as I noted previously when discussing the effects of ischemia on the kidney of cats perfused with 4 M glycerol, fracturing occurs in solutions or tissues that vitrify (even partially) when they are cooled to below Tg. As you can see in this slide such fracturing is quite dramatic when seen in a flask of transparent vitrification solution. It is even more dramatic when it happens to the brains of humans who are cryopreserved using significant concentrations of glass forming cryoprotectants, of which glycerol is one.

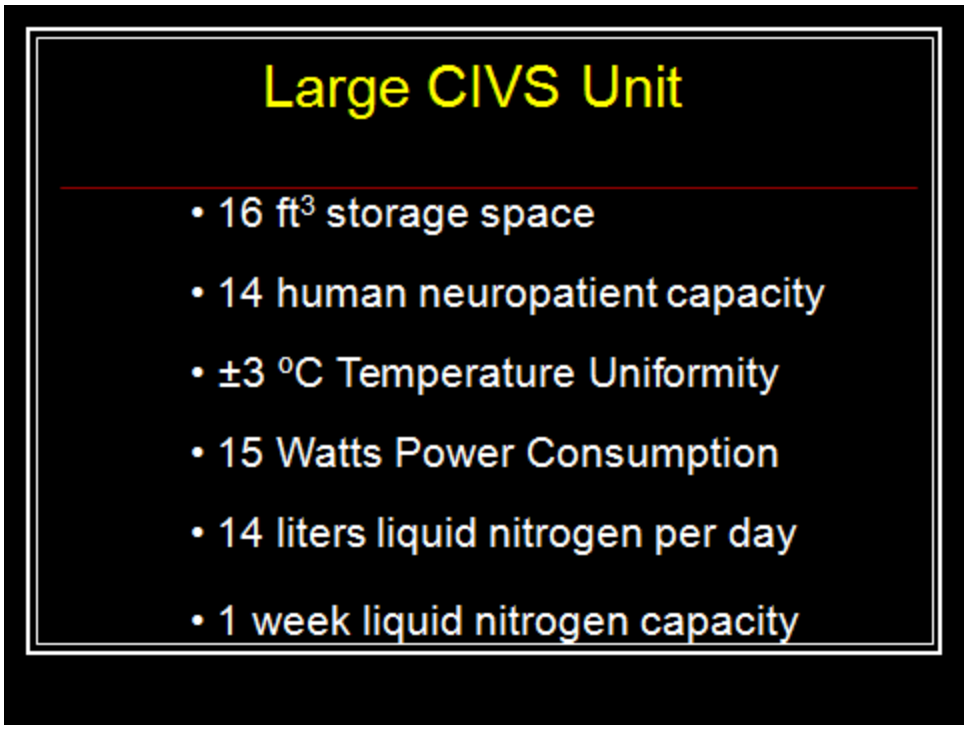
SLIDE 226

In fact, the temperature and the degree to which fracturing occurs upon cooling to below Tg is in large measure a function of the glass forming ability of the cryoprotectant agent(s) with which the tissue has been treated. What this means for vitrified patients is that they will experience more serious fracturing than frozen patients.

The Tg of the vitrification solutions now in clinical use are in the range of ~ -120 to -135^{o}C which would suggest that a relatively ‘safe’ storage temperature will be in the range of ~ -135 to -145^{o}C.

SLIDE 227

21CM has made considerable advances in intermediate temperature storage technology (ITS) which should allow for a large reduction in the number and extent of fractures that occur during cooling to storage temperature.

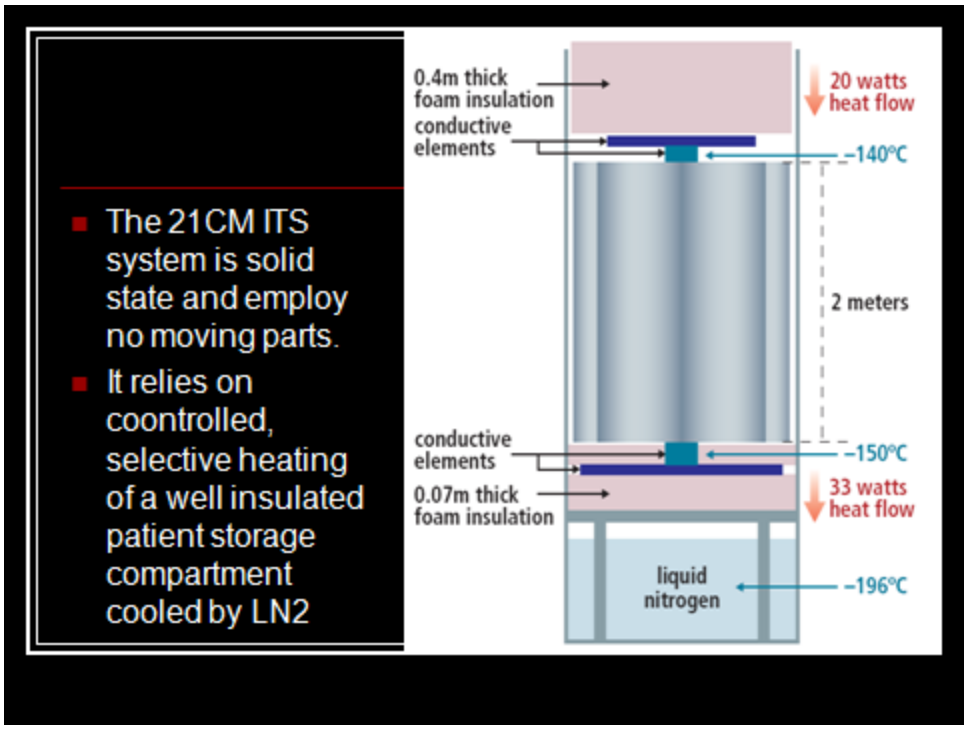


SLIDE 228

Work has proceeded to the point that the first generation of safe and reliable ITS systems capable of storing 14 neuropatients has been fabricated and tested.

SLIDE 229-230

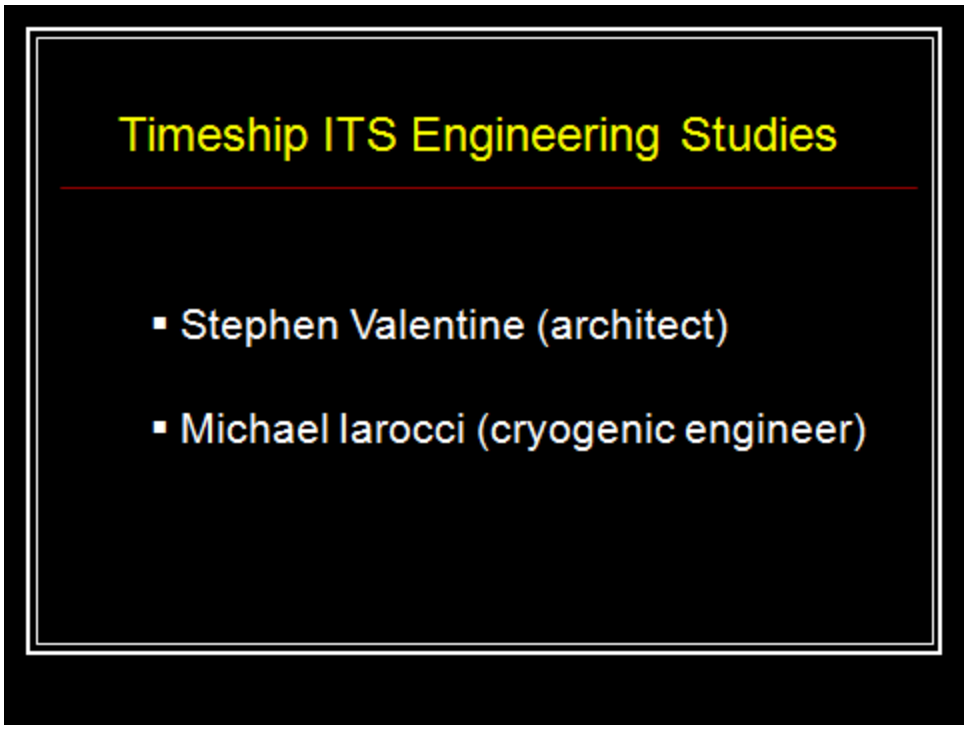
These units can hold 14 neuropatients at any temperature desired between -160^{o}C and -100^{o}C.



SLIDE 231

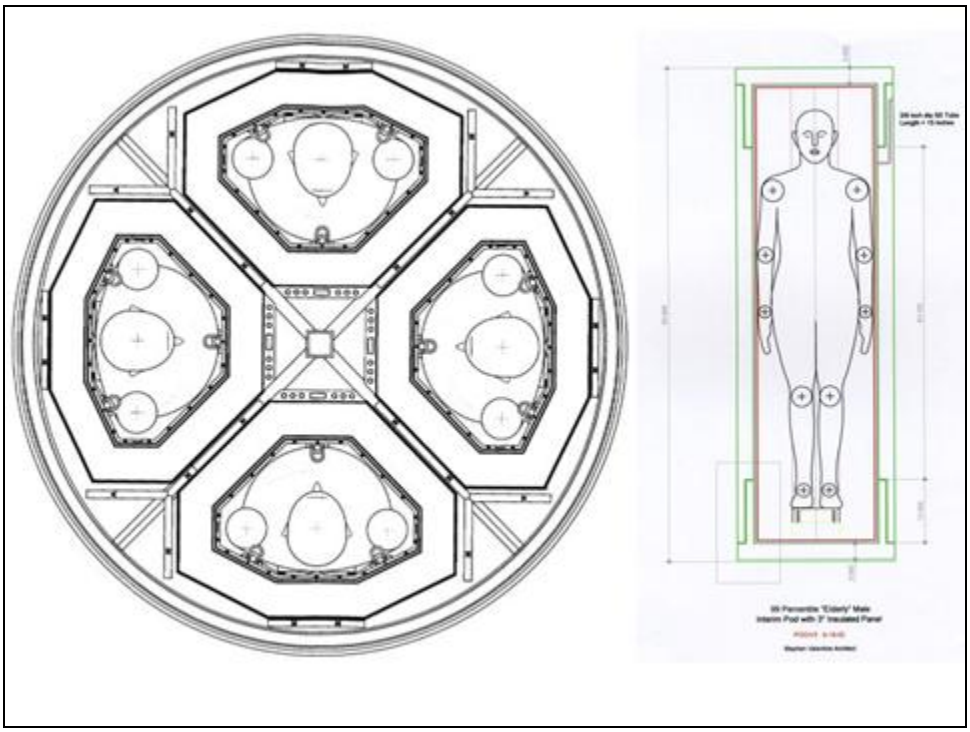
The ITS system developed by Brian Wowk at 21CM works by storing the patients in a highly conductive and well insulated chamber contained within a standard high vacuum cryogenic dewar. Liquid nitrogen provides the refrigeration and compact, solid-state heaters maintain the temperature in the patient storage chamber at the desired temperature within a fraction of a degree C.

And yet these units are not being used by Alcor, or any other cryonics organization even now, 4 years after they were first validated! As a result, cryonics patients continue to be subjected to extensive injury which is either largely or completely avoidable.



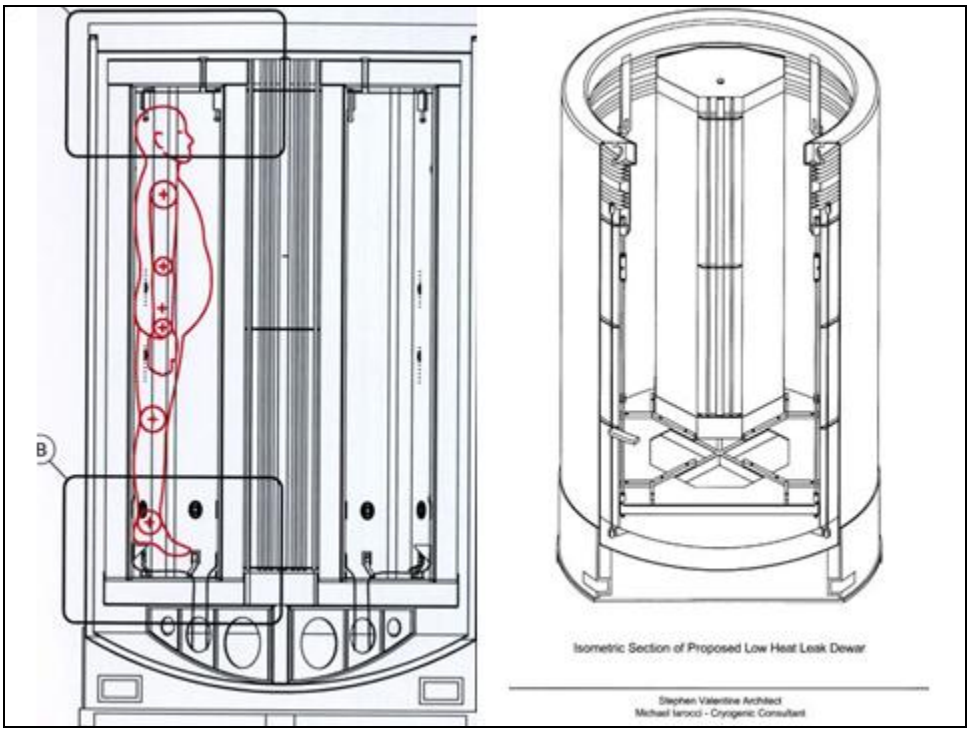
SLIDE 232

While the system developed by 21CM for ITS storage is currently only available for neuropatients, the Timeship ITS Engineering Studies program has produced detailed designs for a whole body ITS system.



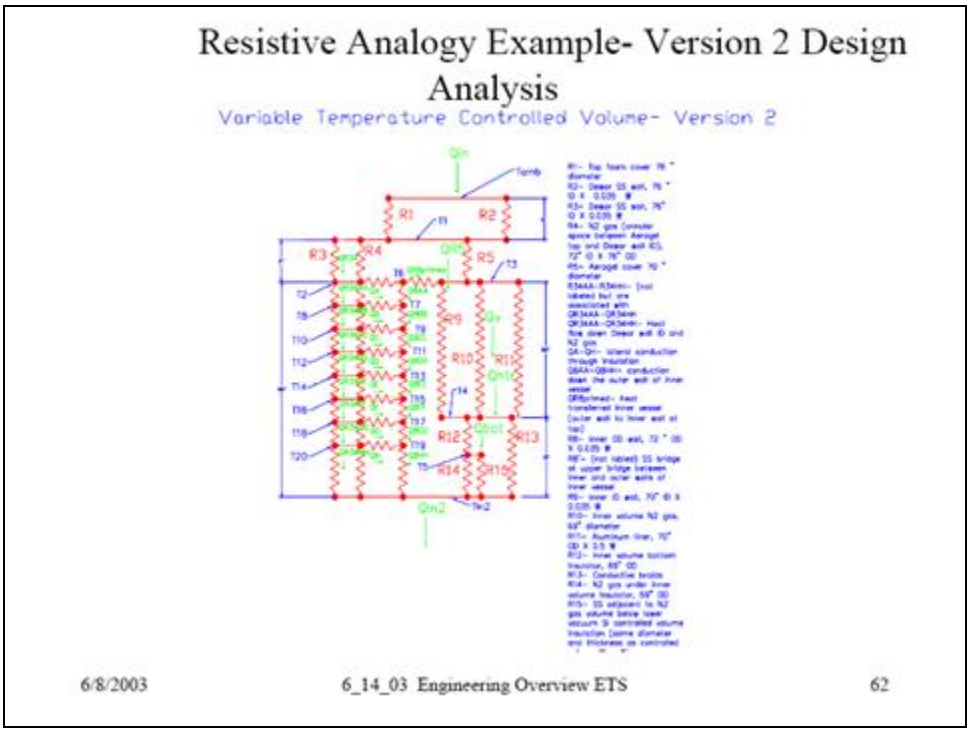
SLIDE 233

This work, carried out by Stephen Valentine, Michael Iarocci and Brian Wowk has yielded sophisticated engineering designs for ITS units capable of holding whole body patients.



SLIDE 234

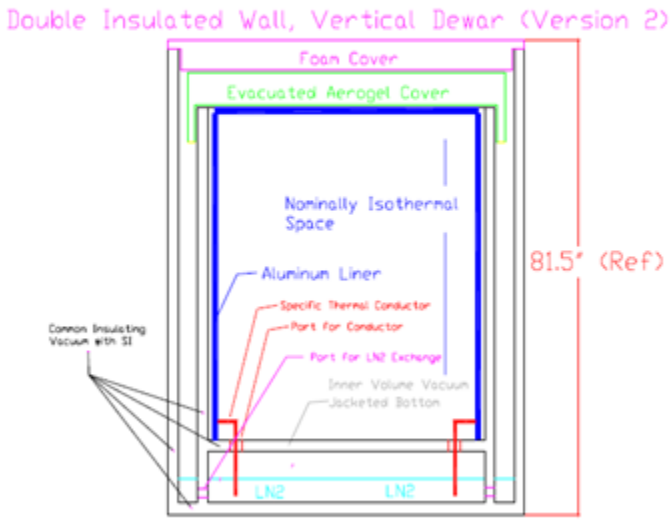
This design allows for storage of 4 whole body patients and the usual ‘bigfoot’ allotment of neuropatients or companion animals.



SLIDE 235

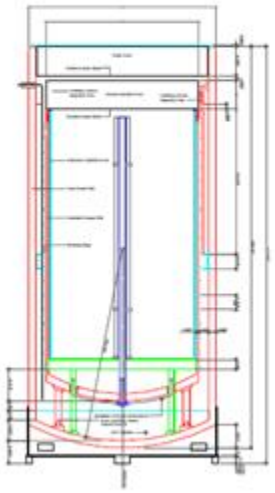
The circuitry used to control the heaters is elegant and simple – and is so inexpensive it can be duplicated as many times as is necessary to assure reliability.

Temperature Controlled Volume Concept



6/8/2003 6_14_03 Engineering Overview ETS 55

TCV Version 4 Design

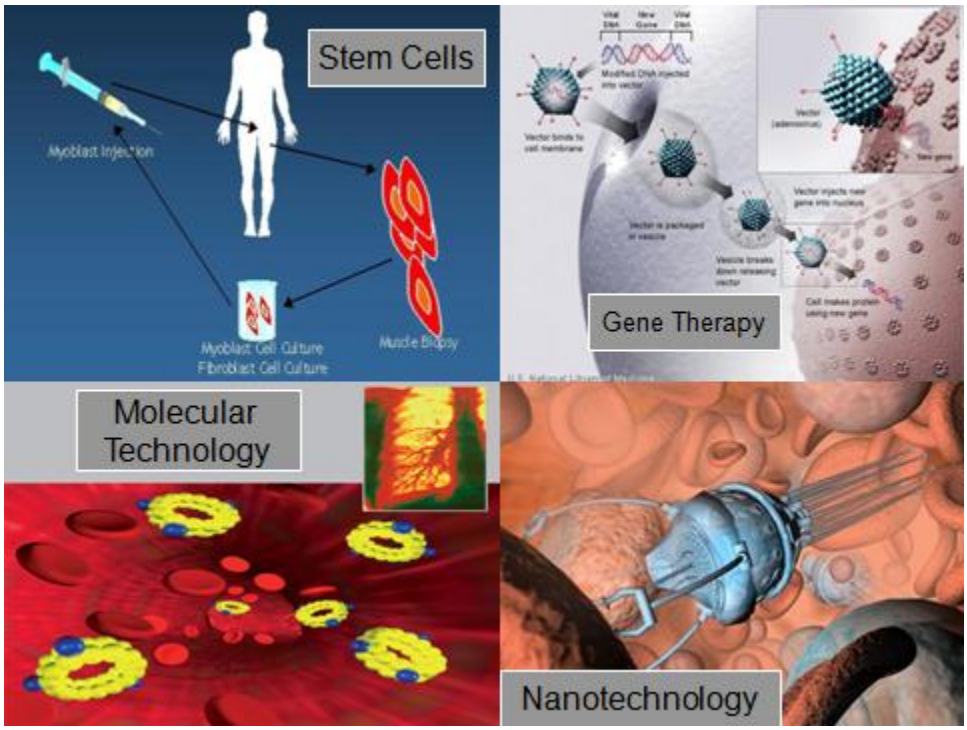


6/8/2003 6_14_03 Engineering Overview ETS 60

SLIDES 236-237

The temperature controlled volume concept, invented by Brian Wowk, provides for a robust, safe and passive

system for ITS which has no moving parts and is fail-safe, in that failure of the control system will result in further cooling (and fracturing) of the patient, not warming and thawing.



SLIDE 238

Summing up, the technology now exists to cryopreserve humans in a high state of both macro and micro-structural integrity. For the first time we are in a position to be able to state, with confidence, that patients treated under optimum conditions will be indefinitely preserved in a condition where the gross and microscopic injury is both well quantified and qualified – and is foreseeably reversible by technologies that are emerging now. And yet it is as completely out of reach as it was that

night in January, 1967 when Dr. Bedford was cryopreserved over 40 years ago.

END PART III

ACKNOWLEDGEMENTS

Bill Faloon

I would like to thank the following individuals and organizations for their contributions, intentional or otherwise, to this lecture:

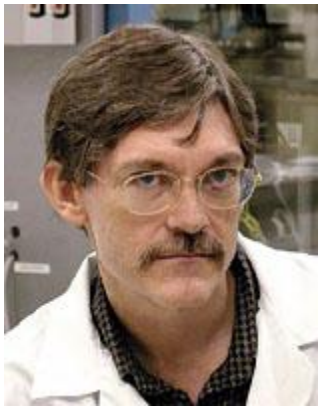
Wherever the name 21st Century Medicine (21CM) appears, it should be understood that Saul Kent is being credited, in whole or in part, for his enormous contributions to that enterprise. Saul, along with Paul Wakfer and me, was one of the founders of 21CM and he has been the principal person responsible for the executive management of the company and for funding allocation for the company's myriad activities since 1996. On par with Saul is Bill Faloon, whose tireless, grinding and oft unappreciated highly creative efforts are responsible for generation of the fountainhead of the Life Extension Foundation's (LEF) largesse. Without LEF there would be no 21CM.

Saul Kent

Brian Wowk

I would like to thank Brian Wowk, Greg Fahy, and 21CM for making available many of the slides and other images that I have used in discussing evolving vitrification technology. Brian, Greg, and 21CM freely provided scientific materials and supporting images for the education of the public – a commendable and increasingly rare act for both industry and NGOs today. As is the case when this is done for journalists or the media, how these images are used is not their responsibility. I am

Greg Fahy



also at pains to point out that they are in no way responsible for how these images have been used here, or for the opinions and philosophy articulated in this lecture: those are mine and mine alone.

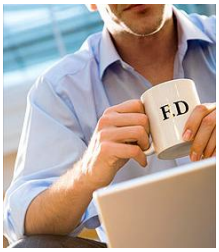
I would also like to thank the Alcor Life Extension Foundation for their forbearance and considerable logistical and practical efforts in allowing the examination of three of their neuropatients in 2002, as well as the capture and use of the images generated as a result of that effort. Similarly, I wish to individually thank Dr. Jerry Lemler, David Shumaker and Charles Platt for their efforts with respect to this project. Special thanks to Charles Platt are in order for his superb photographs taken under difficult conditions.

David Wood

Richly deserved thanks must also go to David Wood, of the ExtroBritannia group in London, UK for offering me the opportunity to present the nascent version of what was to become this (and its accompanying) lectures in 2008. And that brings me to Eugen Leitl, without whom these lectures would not exist. Eugen relentlessly poked, prodded and deviled me into committing that initial lecture to paper, and encouraged, me to expand and refine it.

Eugen Leitl

FD deserves thanks for his proofreading of the lectures and his occasional and critically important suggestions that proved invaluable in ways that probably only he and I will understand.



Maude

Finally, and this may seem strange, I wish to thank the many animals, and especially the dogs and cats, whose lives were taken to make much of the research discussed here possible. I do not believe in 'intrinsic' animal, or for that matter, intrinsic human rights. To do so would require belief in a deity or a teleological universe, and I have no such beliefs. Having said that, I would note that the differences between dog and man are largely cognitive (i.e., intrinsic to the process of acquiring knowledge by the use of reasoning, intuition, and perception) and that raises an important question about what it is that we value most about our humanity?

We generally do not select our mates, our life partners, or our friends and companions based upon their ability to solve partial differential equations or for their encyclopedic knowledge of history – or for skill at any other intellectual undertaking, for that matter. Rather, we choose our companions in daily life mostly based on their decency, fidelity, loyalty, affection, and above all, for their ability to read our moods and feelings, and respond with the affirmation of shared joy, or the balm of sincere comfort, as appropriate.

Dogs, and to a lesser extent some of the other domestics animals with which we share our lives, are true geniuses at this – vastly better at it than are most of our human cohorts to whom we

assign the powerful ‘rights of man’ – and who so often disappoint us by failing to return the favor.

Nevertheless, the statement, “A rat is a pig is a dog is a boy” is a lie, and the fact remains that we humans are at once both ‘more’ and ‘less’ than the animals whose lives we take in order to survive. It is a complex issue, but it is clear, or should be, to any truly perceptive human being that, at their best, the union of intellectual and emotional intelligence represented by humanity transcends that of all other species we have so far encountered.

Cerberus & Pup

This fact does not give us license to kill and maim at will. Nevertheless, it remains an absolute fact of our current and foreseeable existence that we must kill in order to live. The fatuous arguments of the animal rights advocates fails utterly to take into consideration that the acts any species engage in in order to survive necessarily cost the lives of the individuals of other species (and sometimes result in the extinction of entire species). Even the purest vegan existence displaces and kills animals from land under cultivation and – were it possible (which it is not) – the ‘simple’ act of gathering food (removing hunting from consideration) is an act of competition that will leave some other creature the starving loser. The only Eden that has ever existed was in the mind of man, and if such a place is ever to be realized, it will take hard work and the destruction of countless lives achieve it.

That is, in fact, the first half of the message of biological evolution and natural selection: of all the trillion trillon plus living things that have ever existed on this planet, all but a tiny fraction have died in the process of creating those that now exist, including you and me. The second part of that message seems to be that life

has now arrived at a point, after ~4.5 billion years of evolution, where natural selection may, mercifully, come to an end, or at least be attenuated by the expedient of rational design.

So far, we humans are the only candidates on the scene to end the billions of years of mindless slaughter that has been (and is) biological evolution and replace it with thoughtful, rational design – and with compassion. That does not mean there will be an end to the death of organisms, but rather that such deaths may, for the first time, be made more just by the application of knowledge, wisdom and compassion.

To those many animals whose lives I have taken, I would like to offer my sincerest thanks. While there is nothing I can do now to redress your suffering or your loss, I will not forget you, and if, in the fullness of time it becomes possible to redress those injuries and losses, I will act to do so to the extent that fate and physical law allow.

Mike Darwin