Human Cryopreservation Research at Advanced Neural Biosciences

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Introduction

In 2008 we obtained modest funding to establish a laboratory aimed at researching cryonics. Our first challenge was to establish a research program that (a) would distinguish itself from other research labs engaged in cryobiology research, and (b) would be feasible in terms of limited financial resources and time. We immediately recognized that our greatest contribution would be to investigate cryonics protocols under realistic conditions. In this article we introduce the reader to some of our most important and robust discoveries.

Until cryonics becomes available as an elective medical procedure, all cryonics patients will experience varying degrees of cerebral ischemia. Even in “good” cases where stabilization procedures are initiated promptly after pronouncement of legal death, the agonal period prior to cardiopulmonary arrest can give rise to cerebral perfusion impairment. In the case of cryonics organizations that do not offer standby and stabilization services, we should expect at least 24 hours of cold ischemia for a typical (remote) patient, often preceded by significant periods of warm ischemia due to no, or slow, cooling.

The fact that no cryonics patient can completely escape some degree of cerebral ischemia forces cryonics organizations to deal with a fundamental question: how do our protocols and vitrification solutions perform under such conditions? In particular, in our lab we have been interested in the behavior of vitrification solutions in ischemic brains. It should not be a priori assumed that vitrification solutions preferred for non-ischemic tissues are preferred for ischemic tissues as well. A related line of research is whether the composition of carrier solutions can be altered to improve cryoprotectant perfusion in the ischemic brain.

The investigation of cryonics procedures under realistic conditions is by no means exhausted by conducting experiments under ischemic conditions. Another major difference between cryobiology experiments conducted in the laboratory and the practice of cryonics is that the control over perfusion temperatures is limited in cryonics cases. Even the most sophisticated cryonics protocols expose the brain to toxic concentrations of the vitrification agent at high sub-zero temperatures. Thus, our earliest investigations in 2009 were concerned with the effects of exposing red blood cells to high concentrations of VM-1 (the vitrification agent of the Cryonics Institute) in order to address the possibility that exposing a patient to high concentrations of this agent in the absence of rigorous temperature control could produce instantaneous red blood cell lysis (i.e., hemolysis).
**The Red Blood Cell as a Model for Cryoprotectant Toxicity**

Various approaches are available to investigate cryoprotectant toxicity, ranging from theoretical work in organic chemistry to cryopreservation of whole mammalian organisms. One simple model that allows for “high throughput” investigations of cryoprotectant toxicity uses red blood cells (erythrocytes). Although the toxic effects of various cryoprotective agents may differ among red blood cells, other cells, and organized tissues, positive results in a red blood cell model can be considered the first experimental hurdle that needs to be cleared before the agent is considered for testing in more advanced models. Because red blood cells are widely available for research, this model eliminates the need for animal experiments for initial screening studies. It also allows researchers to investigate human red blood cells. Other advantages include the reduced complexity of the model (packed red blood cells can be obtained as an off-the-shelf product) and lower costs.

Red blood cells can be subjected to a number of different tests after exposing them to a cryoprotective agent. The most basic test is gross observation of the red blood cells in a cryoprotectant solution. When high concentrations of a cryoprotectant are introduced (such as in vitrification), a stepwise approach is necessary to avoid osmotic damage. If a cryoprotectant solution is extremely toxic rapid hemolysis will follow, which can be observed as a noticeable change of the color of the solution, hemolyzed cell debris sinking to the bottom of the test tube, or negligible difference between the pellet (if there is one at all) and the supernatant after centrifugation. It is important to keep in mind that these effects only indicate gross membrane damage and that absence of hemolysis is not equivalent to absence of cryoprotectant toxicity.

In our investigations we did not observe instantaneous hemolysis of sheep red blood cells when 70% of VM-1 (in carrier solution) was introduced in a stepwise fashion either at room temperature or close to the freezing point of water. Morphological studies with light microscopy showed slight alterations for VM-1 (dehydration, decreased uniformity) but we have not seen the extreme alterations and destruction that have been observed in solutions that were formulated to produce hemolysis. Eliminating the step-wise approach and exposing the red blood cells to 70% of VM-1 at once, however, did produce hemolysis. This effect was more pronounced at lower temperatures, presumably because at low temperatures the rate of diffusion of cryoprotectants is further depressed than the rate of diffusion of water, causing more pronounced osmotic damage.

VM-1 consists of 35% dimethyl sulfoxide (DMSO) and 35% ethylene glycol (EG). Cryobiologist Yuri Pichugin identified this binary cryoprotectant as one of the least toxic (non-patented) binary vitrification solutions for the vitrification of rat hippocampal brain slices. DMSO is a stronger glass former than EG, but in the case of DMSO as a mono-agent, stepwise exposure of red blood cells to a 70% solution produced complete instantaneous hemolysis. This observation corroborates the contribution of specific toxicity to hemolysis of red blood cells and the need for toxicity neutralization in vitrification solutions.
Since red blood cell hemolysis assays are not optimal for quantifying minor differences in cryoprotectant toxicity, or for investigating the effects of cryoprotectants on organized nervous tissue, we limit our use of this method to preliminary investigations of new variants of VM-1 and/or alternative carrier solution composition.

**Perfusion of the Ischemic Brain**

The brain distinguishes itself from most other organs by its high energy utilization. When the brain is deprived of oxygen and other energy substrates, a complex biochemical cascade ensues that ultimately results in decomposition. Since we do not know the degree of degradation that still permits meaningful reconstruction of the original state of the brain, the most conservative approach is to limit ischemia as much as is practically possible.

The human brain is too large to use immersion as a method to replace water with a cryoprotectant. This fact necessitates the use of vascular perfusion to prepare the brain for exposure to cryogenic temperatures. As a consequence, the ability to protect the brain against ice formation is not an independent challenge but depends on the state of the brain at the time of cryoprotective perfusion. It is at this juncture of ischemia and cryoprotective perfusion where we have conducted most of our experiments.

In a non-ischemic brain, sub-optimal equilibration of the vitrification solution may be compensated by dehydration. This phenomenon is of limited relevance to patients with extensive cerebral ischemia because, as ischemia progresses, the blood-brain barrier through which such dehydration is mediated will become progressively disrupted. For example, cryoprotective perfusion of the non-ischemic rat produces severe dehydration of the brain. After 24 hours of cold ischemia, this dehydration is sharply reduced, and after 48 hours there is no evidence of cerebral dehydration after cryoprotectant perfusion. This phenomenon allowed us to investigate cryoprotective perfusion under ischemic conditions without modifications to the carrier solution to limit cryoprotectant-induced shrinking of the brain.

Our first approach to study the effect of ischemia on perfusion impairment in the brain was to add India ink to the perfusate. Areas with no, or poor, perfusion are distinguished by residual blood and absence of ink. In those studies we limited ourselves to investigating the perfusability of the brain without subsequent freezing to obtain a basic understanding of this phenomenon without additional variables.

Inspection of the brain after ink perfusion showed that 60 minutes of ischemia at room temperature is sufficient to produce noticeable perfusion impairment with the degree and distribution of the impairment worsening progressively as the duration of warm ischemia increases.

Two interventions that are presumed to mitigate perfusion impairment are antithrombotic therapy and induction of hypothermia. Administration of the anti-coagulant heparin prior to ischemia and
the thrombolytic streptokinase following ischemia failed to improve perfusion. This outcome corroborates that ischemia-induced “no-reflow” is not confined to blood clotting and suggests a role for the involvement of blood in a non-coagulating fashion. Scientific and clinical reviews of the no-reflow phenomenon have identified several other factors that contribute to perfusion impairment including red cell aggregation, vasogenic and cellular edema, free radical damage, and inflammatory mediators. Some studies, including the cerebral resuscitation studies of Peter Safar and colleagues, have found benefits from a combination of high perfusion pressures and hemodilution. Our studies into such protocols for short periods of ischemia are inconclusive and for longer (>24 hours) periods of cold ischemia we have found that higher perfusion pressures during cryoprotective perfusion increase ice formation after cooling to cryogenic temperatures.

One of our most robust findings is that rapid induction of hypothermia after circulatory arrest mitigates the no-reflow phenomenon. Perfusion impairment was greatly reduced when the brain was cooled in situ using a miniature portable ice bath. The whole-body cooling rate in these experiments exceeded 1°C per minute. Since such cooling rates are not practically feasible during external cooling in human cryopreservation stabilization without an aggressive combination of different cooling modalities, including cyclic lung lavage, we repeated these experiments at a cooling rate (~ 0.18 °C per minute) that is practical for human cryopreservation and observed the same benefits. These findings strongly corroborate the current practice of rapid induction of hypothermia in cryonics and suggest that even modest decreases of brain temperature can significantly mitigate perfusion impairment, even if the reduction in metabolic demand cannot prevent exhaustion of energy in the brain.

Another consistent finding in our research is that blood substitution prior to circulatory arrest strongly reduces perfusion impairment. In the India ink model we did not observe evidence of perfusion impairment after up to 72 hours of cold ischemia following blood substitution with m-RPS-2 (the carrier solution of VM-1). One limitation of this model is that complete washout of the blood prior to ischemia excludes observation of residual blood after perfusion as an indicator of perfusion impairment. Filling of vessels with India ink correlates strongly with the degree of perfusion impairment but it does not rule out the presence of small pockets of poorly perfused areas in the brain. Because India ink perfusion may not completely predict the degree of cryoprotectant equilibration that is possible after warm and cold ischemia, we further refined our model and introduced observation of the degree of ice formation after cryoprotectant perfusion and cooling as an endpoint.

**Cryoprotective Perfusion of the Ischemic Brain**

As a general rule, cryonics interventions aimed at preventing and mitigating ischemic injury are not evaluated with cryoprotective perfusion and ice formation as an endpoint. As a consequence, there is a serious lack of knowledge about the efficacy of cryonics stabilization protocols on reducing ice formation. One of the most valuable research models in our lab has been to conduct cryoprotective perfusion under various conditions of (cold) ischemia. Space limitations prevent
us from disclosing all our findings, but our most important discoveries are discussed below. Most of our investigations into cryopreservation of the ischemic brain have been conducted with VM-1, the vitrification solution of the Cryonics Institute.

The most fundamental and robust finding in these experiments is that the duration of warm and cold ischemia is positively associated with perfusion impairment and ice formation after cooling to liquid nitrogen temperatures. Our studies corroborate the pioneering feline work that cryonics researcher Michael Darwin did in this area with electron micrographs in the 1980s. In the rat brain we have identified a consistent hierarchy of vulnerability to cold ischemia-induced perfusion impairment as revealed by inspection of the perfused brain and signs of ice formation after cryogenic cooling. The following four major areas are ranked by increasing vulnerability:

*Cerebral cortex; cerebral subcortex; cerebellar cortex; cerebellar subcortex.*

We do not have a full understanding of the reason behind this ranking but these findings may be somewhat comforting in light of our current understanding that the most identity-critical information is stored in the cortex of the brain and that the cerebellum may be the least important area in this regard. Notwithstanding this, our research has been aimed at overcoming perfusion impairment and ice formation in patients with extensive ischemic exposure.

We have studied a number of different interventions to improve outcome in cold ischemic brains and the majority of our experiments involved *alteration of the cryoprotectant carrier solution.* We started by adding various non-permeating salts and sugars and high molecular weight polymers to the carrier solution in order to mitigate edema under the expectation that this would improve outcome. This approach did not produce the desired outcome and meaningful reduction of interstitial edema was not observed either.

We did observe improved outcome in terms of reduction of perfusion impairment in the presence of suitable concentrations of the high molecular weight polymers PVP K360, dextran 500, and dextran sulfate 500. We initially attributed these encouraging outcomes to the ability of these polymers to “seal” leaky membranes, although this interpretation seemed to be at odds with the lack of edema reduction observed.

A real breakthrough occurred when we designed a number of solutions that were made *equiviscous* with a dextran sulfate 500 based carrier solution – our most successful carrier solution to date. All these solutions produced comparable results in terms of overcoming perfusion impairment, indicating that the advantageous properties of these higher molecular weight solutions was not specific to their chemical composition but may be mediated through higher viscosity. This interpretation was further corroborated by our observation that we could also produce improved outcome when we conducted cryoprotective perfusion at lower subzero temperatures, which also increases viscosity of the solutions. Protocols that gradually decreased viscosity during cryoprotective perfusion with the aim of taking advantage of the vessel-clearing properties of higher viscosity solutions at the start of perfusion and improved equilibration of the
vitrification solution towards the end of perfusion failed to improve upon protocols in which the viscosity was kept constant (for a given pressure) across all steps.

Contrary to what one would expect from the vast literature on the no-reflow phenomenon, conducting cryoprotectant perfusion at high pressures (>100 mmHg) in brains with 24 and 48 hours of cold ischemia worsened the outcome. We speculate that these high pressures “push” more perfusate with low glass-forming properties into the interstitial space, limiting the equilibration of the higher concentrations of the vitrification solution during later stages of perfusion. As a matter of fact, many of our best results were obtained when we lowered the perfusion pressure below our standard arterial line pressure of 100 mmHg. We also observed improved perfusion and reduced ice formation when we eliminated one or two steps in our three-step perfusion protocol. This finding may offer some important clues to the mechanisms that contribute to improved cryoprotectant perfusion in the ischemic brain. Since starting with such high initial concentrations of the cryoprotectant at the start of cryoprotective perfusion clearly contradicts basic cryobiology practice to minimize osmotic injury and consequent cell rupture, we have not explored this approach in much detail.

So far, we have employed three distinct cooling methods. In our earliest cooling experiments we used liquid nitrogen plunging to cool samples to liquid nitrogen temperatures. To avoid fracturing, we later modified a small lab dewar to allow a more gradual descent of the temperature to -130°C (slightly below the glass transition temperature of VM-1). Currently we employ an ultra-low temperature electrical freezer that can cool samples to -130 degrees Celsius, which also permits us to store our samples for longer periods our time. Our findings concerning ice formation after cryoprotective perfusion of the ischemic brain have been identical for all three cooling methods. The distribution of ice formation generally follows the areas of perfusion impairment observed prior to cooling, which validated the investigations we conducted with India ink. We have not found any benefits for the addition of pharmacological agents to the carrier solution. Our best understanding about cold ischemia-induced cryoprotective perfusion impairment is that two major contributing factors are red cell aggregation (i.e., hyperviscosity) and edema.

Organ Preservation Solutions

Remote blood substitution in cryonics has a number of important (theoretical) arguments in favor of the practice. Replacing the blood with an organ preservation solution extends the period that organs can be received from static storage in clinical organ preservation. The procedure also permits a faster cooling rate in the field than is possible with external cooling alone. The mannitol-based perfusate MHP-2 that is currently used by the Alcor Life Extension Foundation has been developed in a series of experiments where dogs were recovered after 5 hours of asanguineous ultraprofound hypothermia.
Cryobiology researcher Yuri Pichugin has questioned the value of remote blood substitution in cryonics because none of the organ preservation solutions that he tested (including MHP-2 and UW Solution) could maintain viability of hippocampal brain slices for periods that are typical of transport times in cryonics practice. Our own research, however, has been informed by the possibility that remote blood substitution may fall short in terms of preserving viability but could still confer benefits in terms of improving cryoprotective perfusion.

We have compared controls (i.e., no blood substitution) against the following washout solutions: m-RPS-2, RPS-2 and MHP-2; and observed that blood substitution does confer significant benefits in terms of improving cryoprotective perfusion and reducing ice formation. In particular, MHP-2 outperformed the other solutions and has allowed us to conduct cryoprotective perfusion after 48 hours of cold bloodless ischemia with no ice formation in the brain after cooling below the glass transition temperature. Even at 72 hours, ice formation is relatively minor compared to 72 hours of cold ischemia in which the blood is left in the brain, which produces severe perfusion impairment and ice formation. These experiments vindicate the practice of remote blood substitution in cryonics, but also emphasize that the composition of the organ preservation solution matters a great deal.

None of the organ preservation solutions we have tested (including more advanced recent formulations from colleagues) mitigate the severe vasogenic edema that is observed during cryopreservation after prolonged periods of cold ischemia. We have designed a number of experiments to improve upon the formulation of MHP-2 but none of these variants has been successful so far in decreasing edema and frequently produced worse results than MHP-2 in reducing ice formation after bloodless cold ischemia.

**Cryopreservation after Chemical Fixation**

The idea to chemically fix the brain prior to cryopreservation has remained a topic of interest among cryonics advocates. As a matter of fact, this procedure was discussed in Eric Drexler’s classic treatment of molecular nanotechnology, *Engines of Creation*. One argument that could be offered in favor of this procedure is that it halts the development of ischemia in patients with long expected delays between pronouncement of legal death and cryopreservation. For a long time this idea has been met with skepticism because of (unpublished) experimental observations that such protocols risk producing intracellular freezing during cooling. Because the current generation of cryoprotectants is designed to eliminate ice formation altogether we revisited this topic and designed experiments to study the effects of cryopreservation after chemical fixation.

When there is no ischemic delay prior to chemical fixation, chemical fixation still permits cryoprotective perfusion, and no ice formation in the brain was observed after cooling to liquid nitrogen temperatures after up to two weeks of hypothermic storage of the fixed brain *in vivo*. These experiments have been unique in that no whole body edema was observed during cryoprotective perfusion. We did, however, observe severe dehydration of the brain following
cryoprotective perfusion of the fixed brain, a phenomenon we were not able to eliminate when we added an agent to open the blood brain barrier to our carrier solution.

A practical limitation of cryopreservation after fixation is that delays between pronouncement of legal death and fixation could compromise the efficacy of this procedure and produce the kind of freezing damage that has traditionally been associated with this procedure. When we delayed chemical fixation by an hour, washout of the blood and fixation were incomplete and extensive ice formation followed cryoprotective perfusion. This phenomenon may be overcome by alteration of the fixative carrier solution and different perfusion protocols, but it is doubtful that such sophisticated protocols can be realized in most of the cases where the combination of chemical fixation and cryoprotection may be attractive.

Electron Microscopy of the Ischemic Brain

In collaboration with Dr. Michael Perry of the Alcor Life Extension Foundation we have prepared brain tissue samples for electron microscopy for time points up to 81 hours of normothermic ischemia. Since the rat brain cools at a much faster rate than the human brain after circulatory arrest, we decided that using an incubator to keep the in vivo brain at body temperature would be a better and more conservative approximation of what would be expected to occur in human brains. The electron micrographs have given us insight into the ultrastructural properties of the brain after various periods of warm ischemia. Dr. Perry is using these images to develop an algorithm that models the state of ischemic tissue after various periods of warm ischemia.

Dr. Perry has also supported investigations to examine the degree of fixation and long-term effects of delayed fixation of the brain. Preliminary results of these experiments indicate that even short delays between circulatory arrest and chemical fixation of the brain produce incomplete fixation and risk of progressive decomposition of poorly fixed areas over time. Whether such findings discredit chemical fixation as a low cost alternative to cryonics cannot be conclusively resolved by experimental research due to our incomplete understanding of the neuroanatomical basis of identity and the capabilities of future cell repair technologies. One might also argue that a straight freeze is preferable to chemical fixation but that chemical fixation is still preferable to complete decomposition.

Implications for Cryonics Protocols

To date, our investigations into cryopreservation of the ischemic brain strongly support the practice of standby and stabilization in cryonics. In particular, rapid induction of hypothermia after pronouncement of death and remote blood substitution with an organ preservation solution can limit the degree of perfusion impairment and ice formation after cryoprotective perfusion and cooling. We have identified some emerging principles for alteration of carrier solutions and cryoprotective perfusion protocols that can overcome no-reflow in the brain after cold ischemia and reduce ice formation. In patients with varying levels of ischemia, such protocols are still
confined to the experimental stage until ultrastructural and viability assays have validated the use of these solutions and protocols.

Our research suggests that chemical fixation of the brain prior to cryoprotective perfusion could be beneficial in case of prolonged (transport) delays, but adverse effects of ischemia limit the use of such protocols to a very narrow set of circumstances in which there is negligible delay between circulatory arrest and chemical fixation.

**Future Developments**

Future developments in our lab concern further refinements of perfusion and cooling protocols. In our more recent experiments we have been conducting cryoprotective perfusion using an open ramp system that gradually introduces the vitrification agent to the brain (as opposed to distinct steps of increasing concentration) combined with cooling to just below the glass transition temperature of the vitrification solution. We will keep upgrading our cryoprotective setup to make it conform to conventional perfusion equipment; ultimately, we hope to introduce computer controlled features. We also aim to alter our circuit to conduct cryoprotective perfusion at controlled high subzero temperatures.

A major portion of our time and resources in the coming years will be devoted to developing a set of viability assays that can be used to screen the toxicity of improved vitrification solutions. Such assays will not be confined to *in vitro* brain slice work but will include whole brain *in situ* electrophysiology as well.

We have also received financial support to develop a whole body resuscitation model, which will allow us to validate organ preservation solutions and vitrification solutions at hypothermic and high subzero temperatures.

Our effort to simulate realistic cryonics conditions in our lab remains a work in progress. So far we have mostly limited ourselves to cryoprotective perfusion after either warm or cold ischemia, with a strong emphasis on cold ischemia. Recent observations in our lab indicate that there is a distinct pathophysiology associated with warm ischemia (and *hyper*thermia) that limits simplistic extrapolations between cold and warm ischemia using the Arrhenius Equation.

In a more realistic cryonics model variable periods of warm ischemia precede cold ischemia. In particular, we aim to investigate the efficacy of blood substitution when blood substitution is delayed; a scenario that is common in cryonics practice and that basically constitutes the rule for organizations that do not offer standby and stabilization services.

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