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CRYONICS

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3RD QUARTER 2007 • VOLUME 28:3

THE SCIENCE OF CRYONICS

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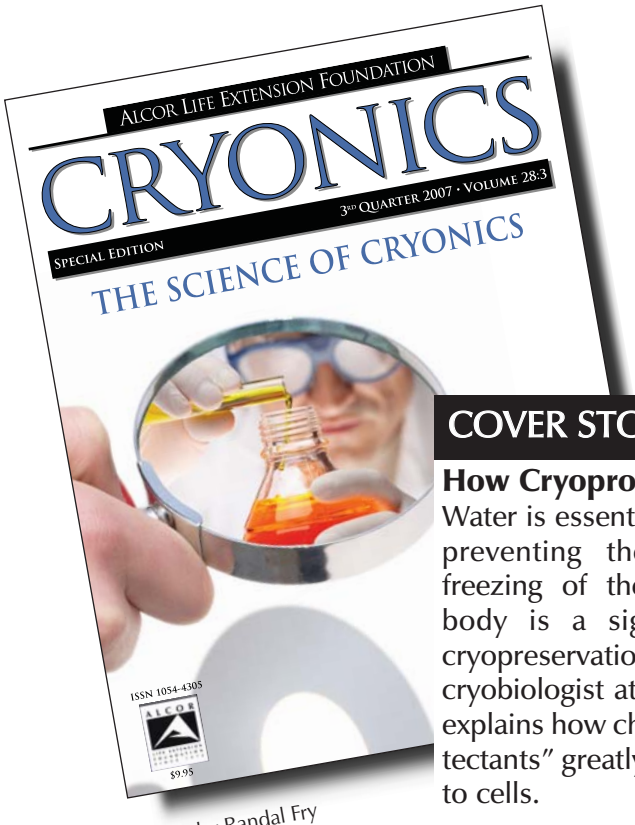

Cover by Randal Fry

COVER STORY: PAGES 3-7

How Cryoprotectants Work: Water is essential for life processes, but preventing the damage caused by freezing of the water in the human body is a significant challenge for cryopreservation cases. Brian Wowk, cryobiologist at 21st Century Medicine, explains how chemicals called "cryoprotectants" greatly reduce freezing injuries to cells.

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CRYONICS

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FROM THE 'GUEST' EDITOR

Chana de Wolf

It has been four decades since the first human cryopreservation was performed. Since then, there have been several advances in cryopreservation techniques and an accumulation of knowledge about the physiological and cellular events after cardiac arrest. As new and relevant discoveries are made, researchers must integrate information from a variety of scientific fields in order to improve cryonics protocol and deliver consistent quality of care. Though the goal of cryonics today remains the same as forty years ago, we have learned (and forgotten!) a lot since then.

Some people think that Alcor's emphasis should be on pushing the science of cryonics forward in terms that scientists will understand. Others believe that Alcor's emphasis should be on outreach and educating its membership. The history of *Cryonics* Magazine reflects both perspectives. The magazine has featured either "dense" technical articles that documented the current state of the art in terms familiar to researchers and biomedical professionals, or articles that appeal to the average reader.

As the guest editor of this issue, I have incorporated both approaches in this "special edition." Most of the usual contents have been stripped to make room for a series of articles that discuss the scientific or technical aspects of cryonics.

There are many misunderstandings about vitrification and the cryoprotective agents Alcor uses. In his article "How Cryoprotectants Work," cryobiologist and Alcor director Brian Wowk introduces the reader to the basics of cryopreservation without ice formation and clears up some of these misconceptions.

Although long term care at cryogenic temperatures is Alcor's current mission, Mike Perry reminds us of the possibility of alternative techniques to preserve critically ill patients in his article "The Road Less Traveled: Alternatives to Cryonics."

We are extremely proud to feature a historical article on the use of DMSO and glycerol by former Alcor president and critical care expert Mike Darwin. On a less positive note, this issue will feature Greg Fahy's final "Advances in Cryopreservation" column. We thank Greg for writing this excellent column and hope to see more of him in the future.

Don't forget that *Cryonics* Magazine has a "Letters to the Editor" section (see left-hand column for contact info)! Let us know what you like and what you don't like about *Cryonics* Magazine.

HOW CRYOPROTECTANTS WORK

By Brian Wowk, Ph.D.

Life is a complex chemical process that happens in water. Without liquid water, there is no life, or at least no life process. Cryoprotectants are chemicals that protect living things from being injured by water freezing during exposure to cold. How cryoprotectants work is a mystery to most people. In fact, how they work was even a mystery to science until just a few decades ago. This article will explain in basic terms how cryoprotectants protect cells from damage caused by ice crystals, and some of the advances that have been made in the design of cryoprotectant solutions.

How Freezing Injures Cells

Water expands when it freezes, but contrary to popular belief it is not expansion of water that causes injury. It is the purification of water during freezing that causes injury. Water freezes as a pure substance that excludes all else. It is this exclusion process that causes injury. Instead of remaining a solvent that allows the molecules of life to freely mix within it, water that freezes gathers itself up into crystals pushing everything else out. This is illustrated in Figure 1.

Freezing causes damage by two distinct mechanisms. The first is mechanical damage as the shape of cells is distorted by ice crystals. The second is damage caused by chemical and osmotic effects of concentrated solutes in the residual unfrozen water between ice crystals. This is so-called "solution effects" injury.

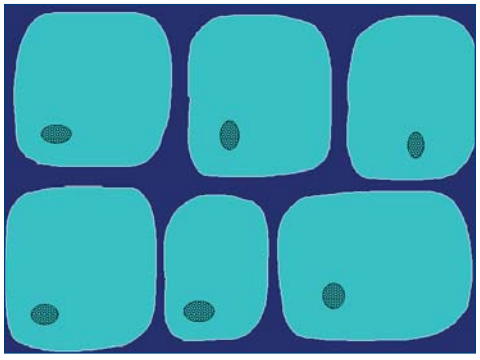

Figure 1A. Cells before freezing.

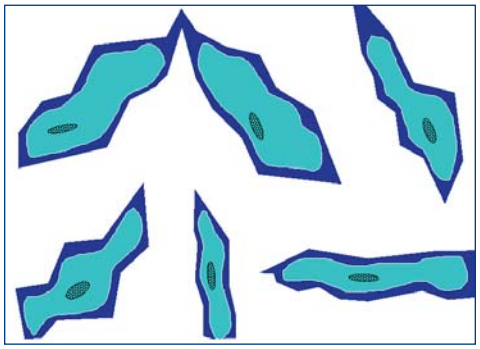

Figure 1B. Cells after freezing. *Cells are squashed between ice crystals and exposed to lethal concentrations of salt. Contrary to popular belief, slow cooling causes water to freeze outside cells, not inside cells. Cells are dehydrated by the growing concentration of salt in the unfrozen liquid around them.*

How Cryoprotectants Protect Cells

Cryoprotectants are chemicals that dissolve in water and lower the melting point of water. For applications outside cryobiology, such chemicals are sometimes called "antifreeze." Common examples are glycerol, ethylene glycol, propylene glycol, and dimethylsulfoxide (DMSO).

A cryoprotectant concentration of about 5% to 15% is usually all that is required to permit survival of a substantial fraction of isolated cells after freezing and thawing from liquid nitrogen temperature. Figure 2 shows the essential concept of cryoprotection during cell freezing. Growing ice compacts cells into smaller and smaller pockets of unfrozen liquid as the temperature is lowered. The presence of cryoprotectants makes these pockets larger at any given temperature than they would be if no cryoprotectant were present. Larger unfrozen pockets for cells reduces damage from both forms of freezing injury, mechanical damage from ice and excessive concentration of salt.

Properties of Cryoprotectants

Not all chemicals that dissolve in water are cryoprotectants. In addition to being water soluble, good cryoprotectants are effective at depressing the melting point of water, do not precipitate or form eutectics or hydrates, and are relatively non-toxic to cells at high concentration. All cryoprotectants form hydrogen bonds with water. Since the discovery of glycerol as the first cryoprotectant more than 50 years ago (1), approximately 100 compounds have been explicitly identified and studied as

cryoprotectants, although only a handful are used routinely in cryobiology (2).

The best and most commonly used cryoprotectants are a class of cryoprotectants called penetrating cryoprotectants. Penetrating cryoprotectants are small molecules that easily penetrate cell membranes. The molecular mass of penetrating cryoprotectants is typically less than 100 daltons. By entering and remaining inside cells, penetrating cryoprotectants prevent excessive dehydration of cells during the freezing process.

Vitrification as an Alternative to Freezing

Organized tissue is more damaged by freezing than isolated cells. Unlike suspensions of disconnected cells, tissue doesn't have room for ice to grow, and cannot easily sequester itself into unfrozen pockets between ice crystals. Organs are especially vulnerable to freezing injury. For an organ to resume function after freezing, all the diverse cell types of the organ, from parenchymal cells to cells of the smallest blood vessels, have to survive in large numbers. The 25% survival rates often seen in cell freezing are not good enough. For cryopreservation of organs, a different approach is required.

In 1984 cryobiologist Gregory Fahy proposed vitrification as an approach to cryopreservation (3). Vitrification, which means "turn into a glass," was previously known in cryobiology as a process that occurred when water was cooled too fast to form ice crystals. It was also believed to be the process by which cells survived in unfrozen pockets of concentrated cryoprotectant between ice crystals at very low temperatures. Fahy proposed a way to turn the entire volume of a tissue or organ into the equivalent of an unfrozen glassy pocket of concentrated cryoprotectant.

To achieve vitrification, it was proposed that the tissue or organ be loaded with so much cryoprotectant before cooling that it could avoid ice formation during the entire cooling process. If cooling is fast, this could be done with actually less cryoprotectant concentration than cells are exposed to during the final stages of conventional freezing. The concept is illustrated in Figure 3.

By avoiding mechanical distortion caused by ice, and by allowing salts and other molecules to remain undisturbed in their natural

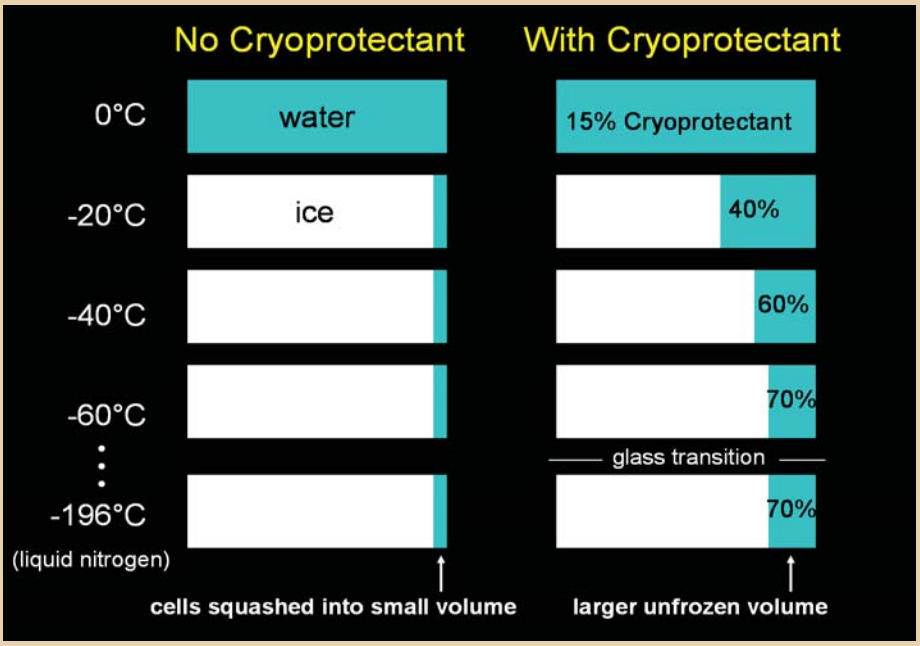

Figure 2. Water when frozen without and with added cryoprotectant. *Without cryoprotectant, almost the entire water volume freezes during cooling. Only salts and other dissolved molecules prevent water from freezing completely. With cryoprotectant, the percentage of cryoprotectant present in solution increases as ice grows. At any given temperature, ice growth stops when the cryoprotectant becomes concentrated enough to make the melting point equal to the surrounding temperature. Eventually the cryoprotectant reaches a concentration that cannot be frozen. No more ice can grow as the temperature is lowered, and there is more room for cells to survive between ice crystals. Below approximately -100ºC, the remaining unfrozen liquid pocket solidifies into a glass, permitting storage for practically unlimited periods of time. Cells survive freezing by existing inside the glassy solid between ice crystals. The larger the starting cryoprotectant concentration, the larger the unfrozen volume will be at the end of freezing.*

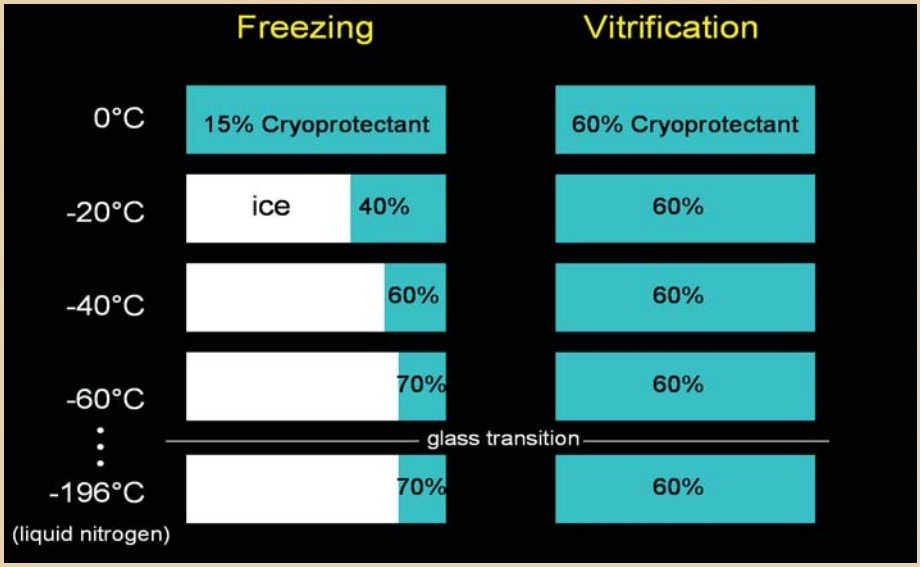

Figure 3. Freezing vs. vitrification. *Vitrification loads cells and tissue with a high concentration of cryoprotectant at the very beginning. Cooling quickly then allows the entire volume of tissue to become a glassy solid, or "vitrify", without any freezing at all.*

locations, vitrification avoids the major damage mechanisms of freezing. The price paid is damage from cryoprotectant toxicity.

Cryoprotectant Toxicity

In cryopreservation by freezing or vitrification, more than half of the water inside cells is ultimately replaced by cryoprotectant molecules. Cryoprotection can be regarded as a process of replacing water molecules with other molecules that cannot freeze. When one considers the crucial role that water plays in maintaining the proper shape and form of proteins and other molecules of life, it is astonishing that this can be survived.

The toxicity of cryoprotectants administered at near-freezing temperatures is a different kind of toxicity than the toxicity experienced by living things at warm temperature. For example, to a person under ordinary conditions, propylene glycol is non-toxic, while ethylene glycol is metabolized into a poison. However at high concentrations near 0°C, ethylene glycol is less toxic to cells than propylene glycol. Usual rules don't apply. New rules relating to how life responds when large amounts of water are substituted at low temperature remain to be discovered.

Mechanisms of cryoprotectant toxicity are still poorly understood (4,5), but a few empirical generalizations can be made. Lipophilicity (affinity for fats and oils) strongly correlates with toxicity. Molecules with an affinity for fat can partition into cell membranes, destabilizing them. It has also been recently discovered that strong hydrogen bonding correlates with toxicity, possibily by disrupting the hydration shell around macromolecules. This led to the unexpected result that cryoprotectants with polar groups that interact weakly with water are best for vitrification, even if a higher concentration is required to achieve vitrification (6). The electrical properties of cryoprotectant solutions have also been related to membrane toxicity (7). Certain cryoprotectants, such as glycerol and possibly DMSO, are also known to have adverse reactions with specific biochemical targets. Finally, mutual toxicity reduction, especially as seen in the DMSO/formamide combination, has been very useful in vitrification solution development, although the mechanism of this toxicity reduction is still unknown (5).

Components of Cryopreservation Solutions

More than just cryoprotectants must be added to cells and tissues to protect against freezing injury. A cryopreservation solution, which may be either a freezing solution or vitrification solution, consists of:

Carrier Solution

Carrier solution consists of solution ingredients that are not explicit cryoprotectants. The role of the carrier solution is to provide basic support for cells at temperatures near freezing. It contains salts, osmotic agents, pH buffers, and sometimes nutritive ingredients or apoptosis inhibitors. The ingredients are usually present at near isotonic concentration (300 milliosmoles) so that cells neither shrink nor swell when held in carrier solution. Carrier solution is sometimes called "base perfusate." The carrier solution typically used with M22 cryoprotectant solution is called LM5.

Different concentrations of cryoprotectant may be required at various stages of cryoprotectant introduction and removal, but the concentration of carrier solution ingredients always remains constant. This constant-composition requirement can be regarded as the definition of a carrier solution. As a practical matter, this means that cryopreservation solutions must be made by means other than adding cryoprotectants to a pre-made carrier solution because naïve addition would dilute the carrier ingredients.

Penetrating Cryoprotectants

Penetrating cryoprotectants are small molecules able to cross cell membranes. The role of penetrating cryoprotectants is to reduce ice growth and reduce cell dehydration during freezing. In vitrification, the role of penetrating cryoprotectants is to completely prevent ice formation. As is shown in Figure 4, penetrating cryoprotectants are the majority ingredients of vitrification solutions.

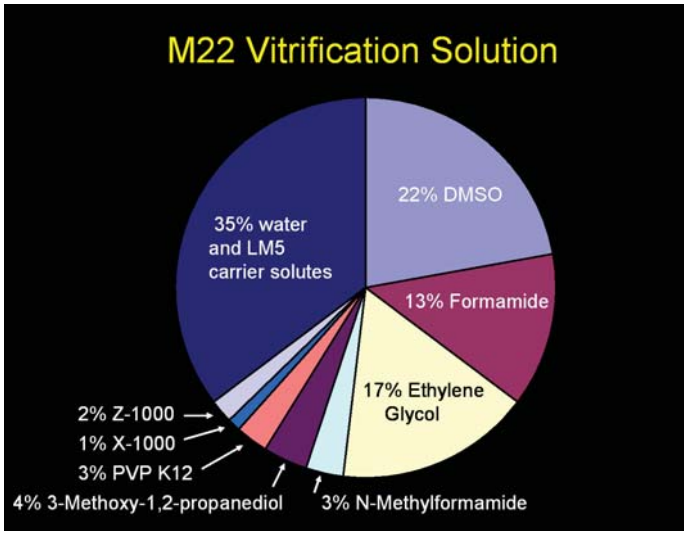

Figure 4. Composition of M22 vitrification solution. *All ingredients are penetrating cryoprotectants, except for LM5 carrier solutes, Z-1000 and X-1000 ice blockers, and PVP K12 polymer. M22 is a "sixth generation" vitrification solution, incorporating two decades of progress in the development of vitrification solutions for mainstream medical tissue and organ banking.*

Non-penetrating Cryoprotectants (optional ingredient)

Non-penetrating cryoprotectants are large molecules, usually polymers, added to cryoprotectant solutions. They inhibit ice growth by the same mechanisms as penetrating cryoprotectants, but do not enter cells. Polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) are examples. Non-penetrating cryoprotectants are usually less toxic than penetrating cryoprotectants at the same concentration. They reduce the amount of penetrating cryoprotectants needed by mimicking outside the cell the cryoprotective effects of proteins inside the cell. It has also been recently discovered that using non-penetrating cryoprotectants to increase the tonicity (osmotically active concentration) of vitrification solutions can prevent a type of injury called chilling injury.

Ice Blockers (optional ingredient)

Ice blockers are compounds that directly block ice growth by selective binding with ice or binding to contaminants that trigger ice formation (ice nucleators). Conventional cryoprotectants act by interacting with water.

Ice blockers compliment conventional cryoprotectants by interacting with ice or surfaces that resemble ice. Ice blockers are like drugs in that only a small amount is required to find and bind their target. Low molecular weight polyvinyl alcohol and polyglycerol, called X-1000 and Z-1000, and biological antifreeze proteins are examples of ice blockers (8,9). Ice blockers are only used in vitrification solutions, not freezing solutions (See Figure 5).

How Cryoprotectants are Used

Freezing solutions containing relatively low cryoprotectant concentrations near 10% are typically added in a single step. This causes the classic shrink-swell response of cryobiology in which cells first shrink by osmosis in response to the high solute concentration outside the cell, and then swell as penetrating cryoprotectants enter the cell. Within several minutes, or tens of minutes for thin tissue pieces, the cryoprotectant concentration inside and outside cells equalizes, and cells return to a volume defined by the tonicity of the carrier solution. The cells or tissue are now ready for freezing. For cryopreservation by freezing, cooling is done slowly, typically less than 1ºC per minute. This allows time for water to leave cells as freezing progresses so that the cryoprotectant concentration inside cells rises together with the concentration outside cells. This prevents cell interiors from freezing. Freezing can also sometimes succeed even though cryoprotectant concentration remains low if freezing and thawing are done extremely rapidly so that there is not enough time for ice to grow inside cells.

Vitrification solutions containing cryoprotectant concentrations near or exceeding 50% cannot be added in a single step because the initial osmotic shrink response would be too extreme. Instead, material to be vitrified is successively exposed to several solutions containing exponentially increasing concentrations of cryoprotectant, such as 1/8 x, 1/4 x, 1/2 x, 1 x full concentration vitrification solution, typically for 20 minutes each step. The addition is done at a temperature near 0ºC to minimize toxicity. The material is then ready for vitrification. For cryopreservation by vitrification, cooling and rewarming are done as quickly as possible.

Unlike cell suspensions or small tissue pieces, organs are too large to absorb cryoprotectant by just soaking in an external solution. For organ cryopreservation, cryoprotectants are added by perfusion, a process in which the cryoprotectant solution is circulated through blood vessels just as blood would flow through the organ. This ensures that no cell is more than a few cells away from contact with the circulating solution. Rather than adding cryoprotectant in discrete steps, it is more convenient during perfusion to increase the cryoprotectant concentration continuously.

Figure 5. Effect of ice blockers on ice formation. *The flask on the left contains 55% w/w ethylene glycol solution that was cooled to -130ºC. The flask on the right contains the same solution, except with 1% of the ethylene glycol replaced by 0.9% X-1000 and 0.1% Z-1000 ice blockers. It is almost completely vitrified, with the majority of the solution being a transparent glass rather than white crystalline ice.*

Cryprotectants are removed by reversing the steps described above, except that all removal solutions except for the very last contain several hundred millimoles of an osmotic buffer, such as mannitol. The role of the osmotic buffer is to reduce the extent of the initial swell response of cells as they are exposed to decreased external cryoprotectant concentration.

Special Considerations for Organs

The time required to introduce and remove cryoprotectants from organs is longer than for cells. For vitrification solutions, perfusion times of hours are typical. This is because cryoprotectants must move through small spaces between cells that line the inside of blood vessels, the capillary endothelium. This makes cells of the capillary endothelium among those most vulnerable to cryoprotectant toxicity because they are exposed to the highest concentrations of cryoprotectant for the longest time while waiting for other cells in the organ to catch up.

The brain has an additional difficulty in that the spaces between capillary endothelial cells are especially small. This is the so-called blood brain barrier, or BBB. The BBB causes penetrating cryoprotectants to leave blood vessels even more slowly than other organs, and doesn't permit water-soluble molecules bigger than 500 daltons to leave at all. Therefore non-penetrating cryoprotectants do not pass through an intact BBB.

However this doesn't mean that non-penetrating agents have no effect on brain tissue. The osmotic movement of water across the BBB is determined by the entire cryoprotectant solution composition. Water moves to equalize the solution melting point, or "water activity," on either side of the BBB. This means that any ingredient that lowers the melting point of the cryoprotectant solution also increases the resistance of tissue outside the BBB to ice formation by drawing out water and increasing the concentration of solutes naturally present in the brain. The brain is an organ in which penetrating cryprotectants and dehydration seem to act in tandem to provide cryoprotection.

Six Generations of Vitrification Solutions

Vitrification solutions have progressed greatly since the initial proposal of modern vitrification by Fahy in the early 1980s. This progress may be viewed as occurring in six generational leaps (10). Generations three through six were developed at 21st Century Medicine, Inc.

Generation 1

The simplest vitrification solutions are single cryoprotectants in carrier solution.

Generation 2

It was discovered that higher total cryoprotectant concentrations with acceptable toxicity

could be achieved by combining DMSO with amides such as acetamide or formamide, and then adding propylene glycol. The combination of DMSO, formamide, and propylene glycol was the basis of the VS41A (also called VS55) vitrification solution, the most advanced vitrification solution of the mid 1990s.

Generation 3

A breakthrough occurred with Fahy's discovery that cryoprotectant toxicity correlated with the number of water molecules per cryoprotectant polar group at the critical concentration needed for vitrification, so-called qv* (6). This led to the replacement of the propylene glycol in VS41A with ethylene glycol, generating the Veg vitrification solution.

Generation 4

The use of polymers in vitrification solutions permitted further reductions in toxicity by reducing the concentration of penetrating cryoprotectants necessary to achieve vitrification.

Generation 5

The use of ice blocking polymers permitted still further reductions in toxicity by reducing the concentration of all cryoprotectants necessary to achieve vitrification. VM3 is a fifth generation vitrification solution (6).

Generation 6

It was discovered that chilling injury, a poorly-understood injury caused by just passing through certain sub-zero temperature ranges, could be overcome by increasing the tonicity of non-penetrating components of vitrification solutions (11). M22, the cryoprotectant currently used by Alcor, is a sixth generation solution.

Successful vitrification has now been demonstrated for heart valves (12), vascular tissue (13), cartilage (14), cornea (15), and mouse ovaries (16, 17). Progress continues for the rabbit kidney, with recovery of the organ demonstrated after cooling to below – 40°C while cryoprotected with a vitrification solution (11), and one reported instance of long-term survival after vitrification (18). Vitrification has also shown utility for viable preservation of diverse tissue slices, including brain slices (19), and histological preservation of larger systems (20).

Future generations of cryoprotectant solutions will have to address many problems that are still outstanding, including molecular mechanisms of cryopreservation failure (21), and especially cryoprotectant toxicity. Cryoprotectant toxicity is emerging as a final frontier of cryobiology. The greatest future breakthroughs in cryobiology may come from better understanding and mitigation of cryoprotectant toxicity. ■

Dr. Wowk is the Senior Physicist at 21st Century Medicine, Inc., a company specializing in the development of technology for cold preservation of transplantable tissue and organs.

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HISTORY OF DMSO AND GLYCEROL IN CRYONICS

By Mike Darwin

In The Beginning: Vitrification or Freezing?

In *The Prospect of Immortality,* the book which launched cryonics, Robert C.W. Ettinger suggests that glycerol might be used as the cryoprotectant for human cryopreservation (1) based largely on the fact that it was the dominant cryoprotective agent (CPA) at that time (1962-64) and most of the positive results with sperm and tissues had been achieved with glycerol. Due largely to the flare and flamboyance of "the Father of Dimethyl Sulfoxide (DMSO*)," Dr. Stanley W. Jacob, who was Assistant Professor of Surgery at the University of Oregon Health Sciences Center Medical School, DMSO entered the public consciousness in a big way in the mid-to-late 1960s (2-4). DMSO's anti-inflammatory, and seemingly incredible skin-penetrating properties, were much talked about.

Sometime between 1966 and 1967, Ettinger asked Dr. Dante Brunol, an Italian national living in the US, to produce a formal, written protocol for cryopreserving cryonics patients. Brunol was a biophysicist (Ph.D.), M.D. and surgeon with experience in cardiopulmonary bypass. Brunol, writing under the nom de plume of Mario Satini, M.D., produced a complicated protocol which, while it has a number of deficiencies, is really quite remarkable, and even visionary in several respects (5). Brunol opens his protocol with the following remarks:

"The writer has always favored supercooling rather than the freezing of humans. Supercooling does not lead to the formation of ice crystals. It should be possible to find methods to store humans at temperatures warmer than -30 degrees C, for five years, the time necessary to protect humans from freezing.

When Professor Ettinger, author of The Prospect of Immortality, asked me to devise a method to freeze humans, at first I declined the offer. In my opinion, only a chemical inducing vitrification could save the cells from (ice) crystal damage."

The First Human Cryopreservation Protocol

Brunol then goes on to explain how vitrification is achieved through ultra-rapid cooling and, without naming it, introduces the idea of the glass transition point of water (Tg), a temperature below which water has become a glass and cannot organize into crystals. He further explains why such ultra-rapid cooling cannot be applied, even to tissues, let alone whole humans. Brunol details his protocol which consists of the following core elements:

1) Immediate commencement of CPR at the time medico-legal death is pronounced, preferably augmented with an artificial airway and high FiO2 (fraction of inspired oxygen in a gas) oxygen administration (15 liters per minute). He recommends at least 30 compressions per minute, with one ventilation every four minutes.

2) Placement of a thermistor in the rectum to monitor body core temperature. The thermistor is affixed to a 10" wooden dowel with straps to hold it deeply in the rectum.

3) Immersion of the patient in a special tub filled with ice and 10% DMSO in water while mechanical CPR continues. The tub supports the patient so that his head remains above the water level allowing manual ventilation to continue.

4) Use of the Westinghouse Iron Heart (a mechanical chest compressor) as soon as possible to continue CPR during cooling. CPR with the Iron Heart is to continue until the patient reaches a core temperature of 15 degrees C, or until extracorporeal cooling using closed-circuit CPB can be commenced via femoral-femoral bypass using a heat exchanger.

5) Inject 2 liters of ice-cold 5% Dextran in an isotonic solution via both internal carotid arteries to hemodilute and cool the brain.

Photo by Ted Kraver

Robert Ettinger (foreground) demonstrates use of the Iron Heart, a mechanical chest compressor.

6) Femoral-femoral cannulation followed by open circuit perfusion (blood washout) of ~20 gallons (80 liters) of heparinized 20% DMSO, 20% glycerol in saline or other isotonic solution at a pressure of 120 mm Hg, and a temperature of between 1 degree and 4 degrees C.

7) Using a fairly complex circuit Brunol demonstrates a good knowledge of physiology, and proposes perfusing the pulmonary circulation by turning on the Iron Heart and pressurizing the venous circulation (via retrograde flow through the femoral venous cannulae) to 20 mmHg at very low flow, while opening the arterial cannulae to allow effluent to exhaust retrograde into a discard-reservoir. Perfusion of the pulmonary circuit is to commence when the patient's temperature reaches 10 degrees C and is to continue for 15 minutes.

8) Preferably, perfusion with the CPA mixture should terminate when the patient's core temperature is -4 degrees C.

9) Brunol was very concerned about interstitial and intracellular ice crystal damage and he proposed vitrifying the cells by initiating ice crystal formation in the vasculature. He proposed doing this by fol-

** The correct abbreviation for DMSO is Me2SO, as per the International Union of Biochemistry and Molecular Biology (IUBMB) – International Union of Pure and Applied Chemistry (IUPAC) Joint Commission on Biochemical Nomenclature.*

lowing CPA perfusion with the perfusion of a quantity of 10% Dextran at near 1 degree C in saline into both the arterial and the pulmonary circulation. The idea was that this solution would start to freeze immediately, before the CPA could equilibrate from the intracellular and interstitial spaces. Ice would thus form first in the vessels and dehydrate the cells to ~30% of their normal volume.

10) The GI tract, plueural space, and peritoneum were to be filled with a solution (apparently) chilled to below freezing consisting of 20% DMSO, 20% glycerol, and 10% ethanol to facilitate core heat exchange. Each pleural space and the peritoneal cavity are to be filled with 1 liter of this fluid. The balance (of up to ~4 gallons as necessary) was to be used to fill the GI tract (upper and lower).

11) Transfer the patient to a container with a perforated bottom to allow the escape of water from melting ice, and pack the body in ice and granular salt: one layer of salt, one layer of ice, etc., to achieve a temperature of -20 degrees C for 24 hours (to allow for maximal extracellular ice growth and intracellular CPA concentration).

12) Transfer the patient to an insulated container and pack in dry ice followed by cooling to -196 degrees C as soon as possible.

Minus the post CPA perfusion of 10% dextran-saline, this protocol would have been vastly better than anything that would be used in cryonics until at least 1979. Brunol was incorrect in assuming that ice formation would start and subsequently outpace diffusion of CPA into the 10% dextran-saline solution. However, his idea of initiating and largely confining ice formation to the large vessels of the vasculature and the body cavities is an intriguing one. Interestingly, the ability to control the location where ice nucleation begins may today be possible by adding the potent ice-nucleation protein produced by the common soil bacteria, *Pseudomonas syringae* (6), to the perfusate in the circulatory system. If this was done *in addition* to ice-blocking polymers, it might allow for considerable ice formation, but only in the form of very small, non-damaging ice crystals (7). Being able to tolerate significant ice formation would decrease the concentration of cryoprotective agents needed for successful preservation, and thus decrease the injury due to cryoprotective agent toxicity.

When James H. Bedford, the first man cryopreserved, died on 12 January, 1967, Robert F. Nelson of the Cryonics Society of California (CSC) had made virtually none of the preparations Brunol recommended. Some DMSO had been acquired, but no carrier solution was available, such as Lactated Ringer's (LR). Similarly, Robert Ettinger had sent Nelson an Iron Heart, but Nelson had not bothered to get oxygen to power it. Thus, Bedford was pin-cushioned with injections of pure DMSO via syringe, with attempts made to inject the DMSO directly into the right internal carotid artery (8).

Glycerol and the Cryonics Society of New York

Largely because of DMSO's almost mythical property of being able to penetrate cells, it seems to have become the CPA of choice amongst cryonicists on the West Coast from 1967 until 1979. By contrast, the people at the Cryonics Society of New York (CSNY), including Paul Segall, Harold Waitz and Curtis Henderson, read Brunol's protocol and decided to try to implement those parts of it that they thought reasonable and practical. Brunol recommended that an Amtec 209 industrial roller pump, with a Zero-Max speed controller, be used to deliver perfusate. Curtis Henderson, CSNY's President, purchased one of these circa 1968 (I still have it to this day).

Zero-Max (mechanical) controllers were the primary way motor speeds were regulated before solid-state electronic controls came into wide use in the 1970s. The Zero-Max is an oil-immersed adjustable speed drive with four or more one-way clutches that move back and forth, each rotating the output shaft a partial turn for each stroke transmission. Zero-Max controls were used extensively control speed before the widespread application of solid state motor controllers in the 1970s.

The bubble trap was designed by a physician associated with CSNY at that time, Dr. Jane Enzman, daughter of the maverick physicist and engineer Dr. Robert Duncan Enzman.

Unfortunately, this set-up was never used on a patient. CSNY used a Porti-Boy embalming machine. Paul Segall modified the Brunol protocol in a number of unfortunate ways. Segall eliminated any attempt at maintaining post-arrest circulation writing, *"No attempt is made to maintain circulation of the blood for the following reason. It has been observed that if the blood flow falls under 70 mm Hg for more than 5 minutes there is irreversible damage (by today's standards) to the cerebral brain centers. Evidence has shown that perhaps this [is] due to the blockage of the microcirculation of the brain (the capillaries become clogged because of the formation of blood clots). In all likelihood, artificial circulation after death could not be started fast enough to reach the cerebral centers (9)."*

Dr. Dante Brunol, 1967

Segall, in contrast to Brunol, apparently never understood the importance of achieving an adequate intracellular concentration of cryoprotectant. Brunol actually does the math and concludes, based on dilution calculations, that the terminal intracellular concentration of CPA will be 22%. Segall's protocol called for an initial flush with 6 liters of ice-chilled heparinized Ringer's Lactate solution for each 30 pounds of body weight (thus, a 150 pound man would be flushed with 30 liters of Ringer's). This was to be followed by a flush of 8 liters of ice-chilled 20% glycerol in Ringer's for a 150 pound man. An additional liter of 20% glycerol-Ringer's was to be used to fill the GI tract. Following this, the patient was to be transferred to a body bag and packed in ice and salt for 12 hours and then transferred to an insulated box and packed in dry ice.

This protocol, which was used on CSNY patients Steven Mandell and Ann Deblasio, would have resulted in negligible concentrations of glycerol in the patient's tissues – levels not even cryoprotective for cells in culture. Failure to use CPR and anticoagulation as soon after cardiac arrest as possible resulted not only in massive systemic clotting, but greatly delayed cooling as well. Segall's rationale for using glycerol as the sole CPA was based on Suda's work with cat brains (10). CSNY had acquired DMSO, but did not use it.

Learning the Hard Way

By contrast, CSC continued to use DMSO, mostly as a 20% solution in Ringer's. There is no documentation of the temperature, pressure or volume of solution used. In the early to mid-1970s there was an extensive round of correspondence and a second attempt to formulate an optimum perfusion protocol. This time it was Dr. Peter Gouras who was chosen for this task. Gouras proposed using DMSO's "extraordinary" permeation qualities to infiltrate the patient with 65% DMSO after Elford and Walter (13) by *soaking* him in progressively higher concentrations of DMSO as the temperature was concurrently reduced (11). This lead Art Quaife, who was both a gifted mathematician and President of Trans Time, to produce a highly sophisticated mathematical analysis of the diffusion kinetics showing that equilibration by soaking the patient in DMSO would take many months even at 0 degrees C (12).

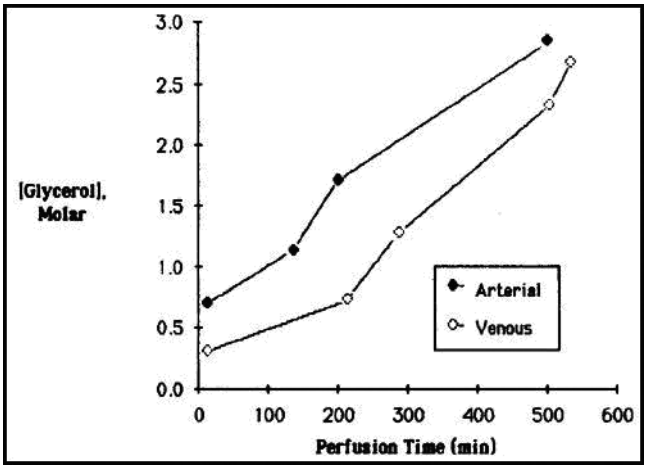
During this extensive collaborative correspondence a consensus was reached to use 20% DMSO in a modified Collin's solution base perfusate, following blood washout with heparinzed Ringer's Lactate. This was the beginning of the end of using DMSO in cryonics. In January of 1973, two patients were perfused on the same day on opposite coasts of the US using DMSO-Collins by Trans Time (San Francisco, CA) and the author (operating as Cryo-Span Midwest) using DMSO-Ringer's (Cumberland, MD) (13). Both patients experienced long periods of warm and cold ischemia before perfusion was possible. Almost immediate and massive edema occurred in both patients with rapid deterioration of venous return, and ultimately, failure of perfusion. Five-percent of DMSO, followed by 20% DMSO in modified Collins solution, was used to perfuse Frederick Chamberlain, Jr., (the first neuropatient) in 1976. Fred, Jr. had been given immediate and continuous cardiopulmonary support, as well as good external cooling. While edema was slower to develop, it nevertheless occurred, and again resulted in failure of venous return (14).

When Jerry Leaf (Cryovita Laboratories) did his first human case for Trans Time, Samuel Berkowitz, in June of 1978 (15), DMSO was again used, but the quantity of perfusate was small, as was the case when K.V.M. (initials used for privacy) was perfused in December of 1978 (16). In both of these cases, despite the low volume of perfusate, edema was a serious problem. The last case done with DMSO was L.R., a Trans Time neuropatient who was perfused (again with a very small volume of 20% DMSO: ~6 liters) in March of 1979 (17). This patient did not experience noticeable edema, probably owing to the small volume of solution used, prompt post-arrest CPR, and minimal warm or cold ischemic injury since she was transported directly from home by ambulance to the Trans Time facility in Emeryville, CA, where perfusion was carried out.

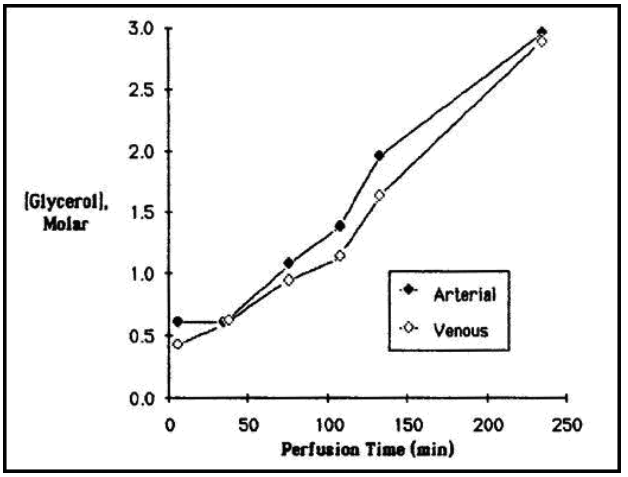
In the summer of 1979, Jerry Leaf and I began intense discussions, both in writing and by phone, about improving the protocol for human cryopreservation. It was during these discussions that the issue of both the CPA to use and the proper volume of perfusate required to reach an adequate tissue concentration arose. I pointed out that the volumes of perfusate being used by Cryovita-Trans Time were only achieving "homeopathic" levels of CPA in the patients' tissues. Jerry was in complete agreement and explained that the CPA protocol he was using had been determined by Paul Segall, of Trans Time.

Clara Dostal, 1973 (Note the lack of edema.)

As of 1977, I had decided that DMSO was unacceptable, because of the consistent problems with edema observed, and its documented destructive effects on the vascular endothelium of kidneys being perfused for attempted organ cryopreservation (18). On 10 December, 1972 Clara Dostal, a CSNY member, was perfused with multiple passes of increasing concentrations of glycerol in Lactated Ringer's (LR) solution in an attempt to achieve multi-molar equilibration of glycerol in the brain (19). Perfusion was via the right internal carotid artery using standard mortuary technique. This meant that only one cannula was available so perfusion had to be alternated between the head and trunk with the arterial cannula being removed and repositioned after each pass of perfusate. The patient's head was flushed with 6.5 liters of LR before commencing cryoprotective perfusion. Three passes of glycerol in LR were used: 2.26 M, 4.34 M, and 5.78 M with concentration on perfusing the brain due to the limited volume of perfusate available. Twenty-seven point two (27.2) liters of perfusate was used with a perfusion time (combined



Graph A. *SP1 Glycerol Concentration vs. Perfusion Time*



Graph B. *SP2 Glycerol Concentration vs. Perfusion Time.*

head and trunk) of 157 minutes. The final cranial (right internal jugular) effluent glycerol concentration was ~4.0 M glycerol.

Despite the comparatively large volumes of perfusate used this patient did not develop edema. (Note: until 1981 this volume of cryoprotective perfusate would have been considered large.) In the winter of 1977, under the auspices of the Institute for Advanced Biological Studies in Indianapolis, IN, I began research on brain ultrastructure following perfusion and freezing of rabbit heads using 2 M glycerol. In November of 1978, I perfused my terminally ill dog, (a ~16 kg mongrel), with 10 liters of 7% v/v glycerol and 30 liters of 20% v/v glycerol. None of these animals experienced edema. Indeed, the problem was systemic osmotic dehydration. On the basis of these experiences it was decided that glycerol would be used in future human cases, with a target terminal tissue glycerol concentration of 3M.

In January of 1980 two consecutive cryopreservation cases (see Graphs A & B) were carried out at Cryovita Laboratories for Trans Time by Jerry Leaf and myself (20). A total of 80 liters of perfusate was used, with the following quantities and compositions:

5% glycerol perfusate, 25 liters
10% glycerol perfusate, 10 liters
15% glycerol perfusate, 10 liters
20% glycerol perfusate, 10 liters
25% glycerol perfusate, 10 liters
50% glycerol perfusate, 15 liters

A combination of open and closed circuit perfusion was used. Incredibly, perfusion was possible in one case for 500 minutes before cerebral edema became the limiting factor. In this patient a terminal venous concentration of 2.32 M glycerol was achieved. In the second patient, a terminal venous concentration of 2.87 M glycerol was achieved after only 133 minutes of perfusion, without either systemic or cerebral edema terminating perfusion.

From that time forward it was clear that glycerol was vastly superior in terms of perfusability. For the first time it was possible to achieve desired levels of cryoprotection, using extended perfusion if necessary, even in patients who had suffered serious warm and cold ischemic injury. In the mid-1980s the target tissue glycerol concentration was increased from 3.0M to 4.5M on the basis of the "Smith Criterion (21)," and, on the basis of dog brain ultrastructural research conducted by BioPreservation in 1995 (22), terminal tissue glycerol concentration was increased to 7.5 M (the maximum concentration possible to perfuse due to viscosity constraints).

An attempt was made by the Cryonics Institute to switch to a perfusate containing propylene glycol in November of 1987 (23). This resulted in severe edema which terminated perfusion well before target CPA concentration was reached. Until the introduction of 21CM vitrification solutions in the summer of 2001 (24), all patients were perfused with glycerol as a mono-agent. ■

Michael G. Darwin was the President of the Alcor Life Extension Foundation from 1983 to 1988 and Research Director until 1992. He was also President of BioPreservation, Inc., and Director of Research of Twenty-First Century Medicine from 1993 to 1999. He is currently an independent consultant in the field of critical care medicine.

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ADVANCES IN CRYOPRESERVATION

By Gregory M. Fahy, Ph.D., Chief Scientific Officer, 21st Century Medicine, Inc.

ADVANCES IN ORGAN FREEZING

I have good news and bad news to report at the top of this installment. The good news is that, as many readers will know, 21st Century Medicine has received a large grant to apply vitrification methods to whole animals. The bad news is that, because of the increased time commitments this new initiative will demand, I will have to make this my last column installment, at least for the foreseeable future. For this reason, I would like to depart from my previous plan and shift the direction of the present installment so as to take this one last opportunity to give due acknowledgement to recent evidence for the contrarian point of view that complex systems can be adequately preserved not by vitrification but by freezing.

Freezing whole ovaries may one day preserve fertility indefinitely. Many people would be affected by success in this endeavor, and the ovary has the large advantage of being something the recipient can live without if it fails.

It is very interesting that, after a very long period of time in which my labs (at the Red Cross, the Navy, and 21st Century Medicine) have been the only labs in the world pursuing the cryopreservation of whole mammalian organs in any serious way, several new groups have become interested in the problem and have published interesting although so far only partially successful results with freezing. Their work is important and deserves to be noted and discussed.

There is a large body of literature on organ freezing from earlier studies, but the only example of the survival of post-transplant vascular function in a whole organ after freezing in liquid nitrogen in the past was provided by frozen-thawed intestines [1]. Now recent events have allowed one more organ to share the intestine's formerly unique status.

The main new initiatives in organ freezing have been efforts to successfully freeze whole ovaries to preserve fertility against the risks posed by chemotherapy in youth or by aging. The ovary freezing effort was kicked off most prominently by a paper [2] that was published in the prestigious scientific journal *Nature* in 2002 by a consortium headed by Roger Gosden, then of McGill University in Montreal, who had a long and prominent previous history of studies on the freezing of ovarian tissue. Seven rat ovaries, still attached to their fallopian tubes and the upper segment of the uterus, were perfused with 0.1M fructose and dimethyl sulfoxide (DMSO) to attain a final DMSO concentration of 1.5M (just a bit over 10% by volume) over 30 minutes, frozen slowly, and stored in liquid nitrogen overnight before thawing, gradual cryoprotectant washout, and vascular transplantation.

Three of seven thawed grafts had no follicles, and the 4 grafts that did have follicles had reduced numbers of follicles and recipients had reduced estrogen levels, elevated FSH levels, and reduced uterine weights, but the 4 surviving ovaries were able to ovulate and one animal was able to get pregnant and have two pups (reduced litter size). This was good enough for *Nature*, and, interestingly, the microscopic structure of the fallopian tubes and uterine portion of the transplants was normal. Clearly, freezing was detrimental, and the authors suggested that vitrification might work better, but just as clearly, at least portions of these ovaries and all of the associated structures survived freezing and thawing.

The next year, investigators at the Cleveland Clinic Foundation and Case Western Reserve University reported freezing and transplanting whole sheep ovaries [3]. Of 11 transplants, only three achieved sustained blood flow although all reflowed with blood immediately after transplantation. Those three transplants that sustained blood flow for 8-10 days permitted normal hormone levels postoperatively, whereas there was "immense tissue loss" and hormone levels were elevated when blood flow did not recover. Follicle survival in the reperfusing grafts was more than 10 times higher than in the occluded grafts, but "scattered areas of necrosis" (dead areas) were still seen even in the patent group, and areas of vascular regeneration were also observed. These results are remarkably similar to the results with rats in that in both cases a minority of ovaries "survived" with damage, but, on the other hand, the fact that any survival at all was achieved is remarkable and positive.

In 2004, Amir Arav's group in Bet Dagan, Israel reported on results of freezing 8 sheep ovaries with a novel directional freezing device (US Patent 5,873,254) using 1.4M DMSO (10% by volume) in Viaspan. This device works by slowly advancing the organ, in a test tube, through a tunnel that is colder at the far end than at the entrance, thereby freezing the organ

from one side to the other. After freezing at 0.3°C/min to -35°C, the ovaries were plunged into liquid nitrogen and later thawed by placement in a 68°C water bath for 20 seconds and then in a 37°C water bath for 2 minutes. Transplantation permitted immediate blood reflow in 5 of 8 grafts, though surgical problems may explain two of the failures. Of the 5 transplants that had immediate return of blood flow, three were able to support normal progesterone levels 34-71 weeks after transplantation, and follicular growth was present at 2 and 3 months postoperatively (overall "survival" rate, 3 of 7 ovaries). All-in-all, these results resemble those of the US group as accomplished without directional freezing.

The same year, a group in Brussels, Belgium, froze three intact human ovaries [4], which are about 10 times larger than sheep ovaries. The only measure of success was microscopic appearance without transplantation, but the ovaries did reasonably well by that standard. Unfortunately, histological (meaning, as seen in the light microscopic) integrity without transplantation generally gives a much better impression of "survival" than evaluation by transplantation.

In 2005, Arav's group reported more details on what seems to be the same set of ovaries described in his 2004 paper [5]. Once again, 8 ovaries were frozen, 5 were transplanted with immediate blood flow restoration, three supported reasonable progesterone levels postoperatively, and other details also matched details in the 2004 report. Two ovaries permitted retrieval of one viable egg cell each one month after transplantation, although 98% of the follicles in the frozen-thawed survivors seemed to be viable based on light microscope examination. Four more viable oocytes were obtained at 4 months post-transplant from one ovary. The histology of the survivors (presumably; no comments were given about non-survivors) was also claimed to be normal. Apparently one of the three ovaries that was able to permit normal progesterone levels in the 2004 study stopped making appreciable progesterone by week 96 after transplantation. A further update on Arav's group's results was presented at the 2007 Society for Cryobiology meeting, but the report was very sketchy and did not seem to contain any particularly critical new information.

All-in-all, these results are significant in that they show whole organs *can* survive freezing, thawing, and transplantation, and that is good to know. However, they all seem to fall very short of clinically acceptable quality standards, and experiments on the vitrification of ovaries have been far more successful [6]. Nevertheless, human clinical trials of frozen-thawed human ovarian transplants are planned at Yale University, and we will have to wait and see how these turn out.

At least two groups have, within the last few years, reported their interest in freezing whole testes informally, but have not published any results. One remarkable report, however, noted that freezing whole testes or whole animals with no cryoprotectant allowed viable sperm to be recovered after thawing [7]! (But then again, even freeze-dried sperm can successfully be used to make babies when injected into good egg cells!)

The last recent example of new interest in organ cryopreservation by freezing was reported by Ralf Dittrich in 2006 [8]. He was able to slowly freeze whole pig uteri to dry ice temperature, thaw them, obtain good contractile responses to drug stimulation, and use these results to challenge the necessity of vitrification [9]! Whether such organs would reperfuse with blood for any length of time in vivo after transplantation is still unknown [10], but it is impressive nonetheless that coordinated muscular action in a large, organized structure like this can be preserved by freezing. Non-freezing approaches can also accomplish the same thing [11], however. So even though freezing can work in some cases based on some measurements and to some extent, preservation without freezing – that is, by vitrification – still seems to be far and away the better option, particularly if what one is trying to preserve really matters.

At 21st Century Medicine, we will continue to focus on developing the best possible methods for preserving what matters the most, and I look forward to communicating with you again about new advances in cryopreservation from our lab as these advances unfold and as the opportunity arises. ■

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DEPRESSED METABOLISM

By Aschwin de Wolf

Snow White: the first "poison-induced" human hibernator

Summary: The most important objective in cryonics after pronouncement of legal death is to drop metabolism as fast as possible to protect the brain and other tissues from rapid deterioration prior to vitrification. To date, most strategies to reduce metabolic demand are of limited value or not practical as an emergency treatment. General anesthetics may only work if they are administered within a very tight time window. Cooling is a potent strategy to protect the brain but presents a number of practical and clinical challenges, including detrimental effects at lower temperatures.

Studies of hibernating animals provide an interesting perspective on the biological mechanisms involved in depressing metabolism and protection from cold injury. So far, practical applications of these studies have been limited to the identification of molecules involved in hibernation and a number of modest successes in organ preservation and reduction of heart and brain injury. Although hibernation on demand for humans remains elusive, recent research into carbon monoxide and hydrogen sulfide-induced "suspended animation" revives hopes for better protection of cryonics patients during stabilization and transport.

Introduction

Human cryopreservation protocol includes two treatment modalities to modulate cerebral metabolism; administration of a general anesthetic and induction of hypothermia. Many general anesthetics are believed to reduce cerebral metabolic demand by reducing excitatory brain activity *via* GABA-A receptor potentiation. It is not surprising, then, that general anesthetics have been investigated as treatments for medical conditions in which cerebral metabolic demand exceeds energy supply, such as focal and global ischemia. Not unlike other neuroprotective strategies explored in ischemia research, results of human clinical trials have been disappointing. This is more remarkable in the case of general anesthetics because reduction of metabolic demand seems to be broader in nature than many other neuroprotective strategies, which target only specific parts of the ischemic cascade of harmful biochemical events that follows cardiac arrest.

Perhaps one reason why general anesthetics ultimately do not improve outcome is that the agent can only be administered during *reperfusion*, when blood supply returns to the tissue after a period of ischemia. However, at this point the energy imbalance has already been upset *during* the ischemic period. Or to state the matter more generally, a neuroprotective agent can only confer significant benefits if the agent intervenes at events that are initiated at or continued downstream from the time of reperfusion. Ischemia-induced cell membrane depolarization is one of the more upstream events that produces a number of different pathological changes (e.g., intracellular calcium overload, mitochondrial failure) that can no longer be reversed by just reducing cerebral metabolic demand with a monoagent upon reperfusion.

Given the extremely narrow time window for mitigating ischemia-induced energy imbalance, perhaps a general anesthetic can only provide a benefit in situations where post-ischemic resuscitation is initiated almost immediately but is inadequate to provide sufficient cerebral perfusion. This situation may apply to a number of in-hospital cardiac arrest situations and, of course, many human cryonics stabilization cases. Although administration of a general anesthetic in cryonics is complemented by a number of other (downstream) neuroprotective medications and fluids, the search for a more potent inhibitor of cerebral metabolic demand remains elusive.

It is tempting to look for an agent that would drop metabolic demand to zero, but considering the fact that most of the energy of the brain is expended on regulating intra- and extracellular ion gradients, reductions in metabolic demand that would interfere with

basic cellular homeostasis without a corresponding drop in metabolic rate will produce ischemic injury themselves. An intriguing question is whether there are broad neuroprotective strategies that are more potent than general anesthetics in reducing metabolic demand but do not upset basic cellular homeostasis or risk damage to the ultrastructural basis of identity and memory. Such strategies would not only include depressing excitatory activity but also inhibiting downstream and intermediate steps in protein synthesis, protein transcription, "futile" repair cycles (such as PARP activation), and immune function. Some of these strategies, or the more radical variants thereof, may still remain largely unexplored in mainstream biomedical research because they would be detrimental in a short-term ischemia-reperfusion recovery model. Examples of these include antioxidant and free radical interventions that are so thorough that they will negatively influence the free radical mediated parts of the immune system after reperfusion.

One obstacle for identifying such strategies is the lack of proper definitions of terms like metabolic demand, metabolic rate and metabolic inhibition. Reducing metabolic "demand" can mean the inhibition of specific physiological events with the result that a number of downstream biochemical reactions are activated at a lower rate, or not activated at all. This is conceptually different from strategies that modulate the rate of biochemical reactions in general, such as induction of clinical hypothermia.

Hypothermia

Induction of hypothermia is an interesting case because it not only reduces the rate of biochemical reactions, but some (patho) physiological are *inhibited* altogether at specific temperatures. For example, humans typically will experience ventricular fibrillation and asystole (no cardiac electrical activity) between 15 and 25 degrees Celsius, whereas some natural hibernators will continue to lower heart rate at temperatures down to the freezing point of water. One reason why hypothermia may even confer neuroprotective benefits when the temperature is only slightly lowered is because some parts of the ischemic cascade, like excitatory amino acid release (excitotoxicity), are inhibited to a greater degree than predicted by the *Q10* value (2.0) that is often associated with induction of hypothermia[1]. Variability in the effects of temperature on protective and pathological reactions may also explain why hypothermia may even be beneficial *after* the ischemic insult.

Induction of hypothermia presents a number of challenges as a clinical treatment. The most obvious challenge is that the human body will attempt to compensate for unnatural drops in temperature by energy-consuming means, such as shivering. In cryonics this may be prevented by administration of a general anesthetic. External cooling also causes peripheral vasoconstriction that further limits heat exchange to the core of the body. Potent alternatives for external cooling in cryonics include extracorporeal cooling and cyclic lung lavage (liquid ventilation).

At very low temperatures (profound and ultra-profound hypothermia), induction of hypothermia itself may produce adverse rheological, metabolic, gastrointestinal, and neurological effects because the balance of protective and pathological metabolic modulation changes in favour of the latter. A good example of this is that cold impairs ATP-driven ion pumps, but passive transport continues as ions move down their electrochemical gradients causing membrane depolarization, intracellular calcium overload, and ultimately, cell death. This is one of the reasons for washing out the blood and replacing it with an "intracellular" organ preservation solution in remote cryonics cases with long transport times.

Although small decreases in brain temperature can confer potent neuroprotective benefits, the logistical challenges of external cooling with ice packs are a concern for cryonics organizations. Not only does the patient need to be enclosed in some kind of portable ice bath to fully benefit from immersion in circulating ice water, effective cooling is also dependent on vigorous cardiopulmonary support and administration of vasoactive medications. Even if cyclic lung lavage can be established promptly as a bridge to extracorporeal cooling, cryonics stabilization could benefit from practical normothermic methods (applied at normal body temperature) of metabolic depression to complement, or as a temporary substitute for, hypothermia. Recent investigations into anoxia tolerance, estivation, and hibernation may guide cryonics-specific research to develop these technologies.

Hibernation

Producing a hibernating state in humans after cardiac arrest seems to be a formidable challenge considering the complex and multifactorial biochemical changes of hibernating animals during the hibernation cycle. Hibernators prepare for dormancy, or torpor, by increasing food intake and storage and by decreasing physical activity. Entrance into torpor is marked by lowering of the hypothalamic temperature setpoint, depression of metabolic activity, sequestering of leukocytes, and a decrease in body temperature. During torpor, heart rate and respirations are substantially reduced, or in the case of *freeze tolerant* animals, like the wood frog, heart rate and respiration are stopped completely[2]. Typical changes in metabolic rate can range from 80% to nearly 100% in cryptobiotic animals, whose metabolic activities come to a reversible standstill. Arousal can be rapid and the need for intermittent euthermic arousal from torpor may involve the need to eliminate sleep debt, restore antioxidant defenses, replenish carbohydrates, and remove metabolic end products.

A number of general criteria apply to all animals that survive long-term hypometabolic suppression: (1) controlled global metabolic rate suppression, (2) storage and alternate energy metabolism and limited production of toxic end products, (3) triggering and signaling transduction mechanisms to coordinate metabolic pathways between cells and organs, (4) reorganization of metabolic priorities and energy expenditure, (5) coordinated up-and-down regulation of genes, and (6) enhanced defense mechanisms such as increased production of antioxidants and stabilization of macromolecules[3]. Hibernating animals prevent hypothermia-induced injury by maintain-

The wood frog is primarily found in the northeastern United States, Canada, and Alaska. In winter, the wood frog freezes 35-45% of its body, and its heartbeat and breathing cease. In the spring, the frog's body thaws with the land around it, and it returns to normal function.

Source: http://en.wikipedia.org/wiki/Wood_Frog

ing membrane potentials, decreasing blood clotting, and limiting energy expenditures to basic physiological necessities at the expense of protein synthesis, gene transcription, and cell division. Selective up-and-down regulation of regulatory enzymes and rapid arousal from torpor is achieved by reversible phosphorylation.

Because aspects of hypometabolism have been induced in some non-hibernating animals by injecting them with the plasma of hibernating animals, some researchers have speculated that a "hibernation induction trigger" (HIT) may exist that controls entry into hibernation. If such a molecule (or number of molecules) exists, it is tempting to think that administration in humans can produce hibernation on demand. Practical applications would range from stabilization of cardiac arrest and stroke victims to long-term space flight. Current investigations into HIT-like substances indicate involvement of opioid receptors.

The most promising HIT mimetic so far is the synthetic delta-opioid peptide DADLE (D-Ala2,D Leu5enkephalin). Administration of DADLE to a normothermic multiorgan block preparation was able to extend survival of organs to 46 hours, including the heart and liver[4]. Using the same multiorgan block autoperfusion method, successful single canine lung transplantation after 24 to 33 hours was achieved when the lungs were preserved with woodchuck HIT-containing plasma[5]. Hypothermic preservation time of the rat lung has been enhanced by adding DADLE to Euro-Collins solution[6]. Improved function of hearts pretreated with HIT or DADLE after hypothermic storage have been reported for a number of non-hibernating species including rats, rabbits and swine[7].

Although beneficial effects of DADLE have been reported in cortical neurons, investigations of DADLE as a neuroprotectant in global and forebrain ischemia have been limited to date. A 2006 study didn't find any improvement for pre-ischemic administration of DADLE in a forebrain ischemia rat model[8]. In 2007 the Safar Center for Resuscitation Research reported that DADLE failed to improve neurological outcome in a deep hypothermic circulatory arrest rat model and even produced worse extracerebral organ injury for the highest dose administered (10 mg/kg). One explanation for these results is poor blood brain barrier (BBB) permeability of DADLE because of its unfavorable hydrophylicity and charge. A series of cyclic prodrugs of DADLE only improved BBB permeability in the presence of a P-glycoprotein inhibitor to prevent P-gP mediated efflux transporter activation. Bioconversion of the parent drug, however, was low[9]. Alternatively, pre-ischemic *cerebroventricular* (ICV) administration of DADLE did confer neuroprotective benefits in a rat model of forebrain ischemia[10]. As these results indicate, neuroprotective agents with high treatment potential do not necessarily have privileged access to the brain.

Opioid receptor modulation in cerebral ischemia has proven to be a viable research direction but the results obtained with HIT-like substances do not seem to produce the multi-factorial and coordinated physiological effects of hypometabolism-mediated cytoprotection that can be observed in hibernating animals. Although induction of artificial hypometabolism in humans may be possible by pharmacologic modulation of conserved metabolic pathways with natural hibernators, it is doubtful that hibernation on demand will be possible anytime soon. This challenge is not dissimilar to cryobiological research that aspires to protect humans from the extensive injury that results from exposure to low (subzero) temperatures. Ultimately, advances in modulation of hypometabolism are necessary to protect cryonics patients from brain injury during stabilization and the descent from normothermia to cryogenic temperatures for long-term care. The subtle adverse effects of exposure of the human brain to low temperatures *as such* may turn out to be one of the final obstacles to be overcome to achieve real suspended animation.

Carbon Monoxide and Hydrogen Sulfide

A number of alternative approaches to induce hypometabolism and hypoxia tolerance that have been explored in recent years include administration of carbon monoxide and hydrogen sulfide. The choice of these two gases is remarkable because both are known to be dangerous poisons at supraphysiological levels. For example, high levels of carbon monoxide can displace oxygen at hemoglobin at a rate in excess of 200 times the rate of oxygen, causing an acute drop in oxygen levels to the tissues. At physiological levels, however, both substances are produced endogenously in the human body where they perform a number of regulatory and signaling functions[11,12]. Therapeutic administration of low concentrations of carbon monoxide and hydrogen sulfide have been investigated in various models of ischemic injury. Low dose

"All things are poison and nothing is without poison, only the dose permits something not to be poisonous." Paracelsus, 1493-1541

carbon monoxide can enhance protection against hypothermic renal injury and improve function of renal grafts[13]. Hydrogen sulfide increases glutathione levels in glutamate-mediated oxidative stress[14].

Of most interest is administration of carbon monoxide or hydrogen sulfide to produce a state of hypometabolism or hypoxia tolerance. *C. elegans* can survive mild hypoxia by hypoxia-inducible factor 1 (HIF-1) modulated anaerobic energy production and up-regulation of antioxidants. *C. elegans* can also survive extreme hypoxia by entering a state of "suspended animation." An intermediate level of hypoxia, however, is deadly to the organism. Carbon monoxide-induced hypometabolism can protect *C. elegans embryos* against this intermediate level of hypoxia, even in the absence of HIF-1 function[15].

Following this line of research, in a widely published series of experiments, hydrogen sulfide has been found to produce hypometabolism and hypoxia tolerance in mice. Although the research to date has not produced much insight into its molecular mechanisms, results presented so far indicate that hydrogen sulfide exposure produces a change in energy utilization and physiological response that is typical of hibernators[16].

Concerns have been raised about the lack of proper temperature controls in these experiments[17]. Although it is typical for real hibernators that reductions in metabolic rate *precede* hypothermia, biochemical versus temperature-induced modulation of metabolism are left implicit in the published results so far. Recent research also indicates that different

strains of mice use different metabolic strategies to protect themselves from acute hypoxia[18]. C57BL/6J (C57) inbred mice, the strain used in the hydrogen sulfide experiments, were found to be more hypoxia tolerant than CD-1 outbred mice.

Even if hydrogen sulfide would not be able to induce profound normothermic hypometabolism, identification of a molecule that could induce a hibernation-like state in humans, or even just confer broad cytoprotection during induction of artificial hypothermia, would be a non-trivial therapeutical breakthrough. The biomedical potential of the gases nitric oxide, carbon monoxide and hydrogen sulfide are currently being investigated by a new biotechnology company called Ikaria, which includes the prominent NO/PARP researcher Csaba Szabo among its scientific staff.

Advocates of human cryopreservation may find the increasing use of the term suspended animation for therapeutic interventions like whole body (ultra) profound asanguineous hypothermia and normothermic hypometabolism indicative of a lack of precision. But the increased support for and research in these areas in mainstream biomedical science and the media may produce a more favorable reception of research aimed at reversible human cryopreservation and real suspended animation as a result. Another advantage of increased research efforts in these areas is that cryonics providers can benefit from these findings to enhance their own capabilities and initiate informed research into improved organ preservation solutions and "hibernation mimetics." ■

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NOSY NEUROPROTECTION:
INTRANASAL DELIVERY OF NEUROPROTECTIVE AGENTS TO THE BRAIN

By Chana de Wolf

Introduction

In everyday or emergency medical practice, intranasal (IN) administration of therapeutic agents (i.e., drug delivery via the nose) offers several advantages over oral, intravenous, and other routes of administration. Drugs can be rapidly absorbed through the large surface area of the nasal mucosa, resulting in a rapid onset of action and avoiding degradation in the gastrointestinal tract and first-pass metabolism in the liver. IN delivery is also non-invasive and essentially painless, which helps to increase patient comfort and compliance. Because of these distinct advantages, explorations into the practical applications of IN administration are becoming increasingly common and IN formulations of a wide variety of therapeutic agents now exist. Nasal administration is a logical choice for topical nasal treatments such as antihistamines and corticosteroids, and the nasal mucosa has also received attention as a viable means of systemic administration of analgesics, sedatives, hormones, cardiovascular drugs, and vaccines[1].

Importantly, the nose also serves as a direct route to the brain. In 1937, W.F. Faber placed Prussian blue dye in the nasal cavity of rabbits and later observed the dye in both the olfactory nerve and the brain, demonstrating for the first time the existence of the olfactory pathway[2]. Several decades later, experimenters discovered the link between the environment and the brain by performing Faber's experiment in reverse: dyes injected into the brain ventricles of rabbits and monkeys showed that the cerebrospinal fluid (CSF) is drained into the lymphatic vessels and the nasal mucosa via the olfactory neurons[3,4]. Thus the olfactory neurons, which function primarily as sensory cells, also provide a means for the direct transport of agents to the central nervous system (CNS).

The implications of direct nose-to-brain transport went largely unnoticed until the 1990s, when enhanced public attention to brain research compelled scientists to discover and implement effective treatment strategies in an effort to combat the upsurge of age-related neurodegenerative diseases and related neurological disorders in an increasingly elderly patient population. A consistent frustration in the treatment of brain disease and stroke is that many drugs known to mitigate the damaging effects of such pathologies are unable to enter the brain from the systemic circulation due to the existence of a tight membranic structure called the blood-brain barrier (BBB). However, because the olfactory receptor cells are in direct contact with both the environment and the central nervous system, the olfactory pathway offers a potential means of circumventing the BBB to deliver neuroprotective agents directly to the brain. Accordingly, IN delivery of drugs targeting the CNS is now an area of great interest[5,6].

In cryonics, certain neuroprotective agents are administered to patients in an attempt to prevent (or at least slow down) ischemic damage to the brain after cardiac arrest and during the low-flow reperfusion provided by cardiopulmonary support. Cooling is, of course, our primary line of defense against such damage because of its striking effectiveness in reducing metabolic demand. However, rapid field cooling still presents considerable logistical and clinical challenges and preferential brain cooling is (at least until the patient arrives in the operating room) yet to be accomplished. Therefore, quick and direct protection of the brain is especially important in the moments following pronouncement and during the initial stages of cooling, while the patient is still relatively warm.

Currently, all medications delivered to cryonics patients are introduced to the systemic circulation either via the intravenous (IV) or intraosseous (IO) routes, requiring skilled personnel who have been trained in IV and/or IO technique. Due to the blood-brain barrier and first-pass metabolism, relatively large volumes of neuroprotective agents must be given in order for an effective dose to enter the brain *via* the systemic circulation. In fact, some of the most promising neuroprotective drugs are currently not available at all for treatment of cryonics patients because of poor BBB permeability.

Additionally, IN administration of cardiovascular drugs has been a growing topic of investigation[7]. IN propanalol provides immediate β-blockade when taken before exercise by patients with angina. IN administration of propanalol exhibits a pharmacokinetic profile similar to IV administration and 10 times greater bioavailibility than oral propanalol (because oral propanalol undergoes considerable first-pass metabolism in the liver). In cryonics, patients may also benefit from IN administration of vasopressors (i.e., medications for the maintenance of blood pressure) during stabilization. IN epinephrine has been shown to reach peak plasma concentrations in only 15 seconds[8], and to improve coronary perfusion pressure in canine models of cardiac arrest and CPR[9]. Indeed, IN administration of epinephrine is a rapidly obtainable and feasible route of administration during any cardiac emergency.

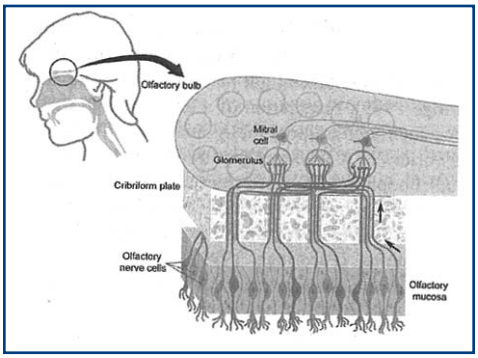

Figure 1. *The olfactory bulb, olfactory mucosa, and olfactory nerve cells in humans. Modified picture from the Nobel Prize offical homepage (Nobelprize.org).*

The Nose

The nasal cavity is split into two symmetrical halves by the nasal septum (comprised of cartilidge and bone) with each side opening at the face via the nostrils and connecting with the mouth at the nasopharynx. The three main regions of the nasal cavity are the nasal vestibule, the respiratory region, and the olfactory region, reflecting the primary nasal functions of vocalization, respiration, and olfaction[10]. The main nasal airway passages are narrow (only 1-3 mm wide), causing inhaled air to come into contact with the nasal mucosa, where particles such as dust and bacteria are filtered by mucociliary clearance. Simultaneously, the air is warmed by the highly-vascularized nasal epithelium and humidified by fluid secreted by the mucosa[10,11]. The epithelial tissue within the nasal

cavity has an extensive blood supply which drains blood from the nasal mucosa directly to the systemic circulation[12], thus providing a potential conduit for drug delivery which circumvents first-pass metabolism.

The Respiratory and Olfactory Epithelium

The respiratory epithelium, making up the middle and posterior thirds of the nasal cavity, can be described as a pseudo-stratified ciliated columnar epithelium consisting of four main cell types: ciliated and non-ciliated columnar cells, goblet cells, and basal cells. The epithelial cell layer is covered with mucus, which is produced by the goblet cells and cleared by the beating of the cilia. This clearance mechanism protects the respiratory tract and lungs from bacteria and other exogenous compounds. In humans, the respiratory mucosa covers most of the total nasal surface area and is the major site for drug absorption into the systemic circulation.

The olfactory epithelium is located at the top of the nose between the superior turbinate and the roof of the nasal cavity, just beneath the cribiform plate of the ethmoid bone (Fig. 1). In humans, it covers only 10-20 cm^2, or about 8% of the total nasal surface area, and is composed of three main cell types: olfactory receptor neurons, supporting cells, and basal cells[13,5]. The olfactory epithelium is more than twice the depth of the respiratory epithelium, with the olfactory nerve cell bodies typically located in the middle and deeper regions of the epithelium while nuclei of the supporting cells are organized in a single layer closer to the mucosal surface. Tight junctions exist between the supporting cells and between the supporting cells and olfactory nerve cells[14].

The olfactory receptor neurons are bipolar neurons that connect the olfactory bulb of the brain with the nasal cavity. The single dendrites of the olfactory cells terminate in olfactory knobs that project above the epithelial surface and exhibit 10-25 immobile cilia which contain receptors for binding odorant molecules. The axons of the olfactory receptor neurons extend from the cell bodies and pass through the lamina propria and cribiform plate as grouped bundles (Cranial nerve I) surrounded by Schwann's cells and perineural cells[3]. After entering the olfactory bulb, the axons synapse with juxtaglomerular neurons and with tufted and mitral cells in the glomeruli[15]. Surrounding brain structures include those of the olfactory area (lateral olfactory tract, olfactory tubercle, and olfactory nucleus) and limbic system (amygdala, pre-pyriform cortex, enthorhinal cortex, hippocampus, thalamus, and hypothalamus)[8].

Nasal Transport Routes

After nasal delivery drugs first reach the respiratory epithelium, where compounds can be absorbed into the systemic circulation utilizing the same pathways as any other epithelia in the body: transcellular and paracellular passive absorption, carrier-mediated transport, and absorption through trancytosis. Although absorption across the respiratory epithelium is the major transport pathway for nasally-administered drugs and may represent a potentially time-saving route for the administration of certain systemic drugs delivered in cryonics medication protocols (e.g., epinephrine or vasopressin), the author considers the problem of BBB-mediated exclusion of brain-therapeutic agents to be of greater immediate concern. Accordingly, the remainder of this article will deal primarily with the transport of drugs to the CNS by way of the olfactory epithelium.

When a nasal drug formulation is delivered deep and high enough into the nasal cavity, the olfactory mucosa may be reached and drug transport into the brain and/or CSF *via* the olfactory receptor neurons may occur. The olfactory pathways may be broadly classified into two possible routes: the *olfactory nerve pathway* (axonal transport) and the *olfactory epithelial pathway*[13].

Axonal transport is considered a slow route whereby an agent enters the olfactory neuron *via* endocytotic or pinocytotic mechanisms and travels to the olfactory bulb by utilizing the same anterograde axonal transport mechanisms the cell uses to transport endogenous substances to the brain. Depending on the substance administered, axonal transport rates range from 20-400 mm/day to a slower 0.1-4 mm/day[16]. The epithelial pathway is a significantly faster route for direct nose-to-brain transfer, whereby compounds pass paracellularly across the olfactory epithelium into the perineural space, which is continuous with the subarachnoid space and in direct contact with the CSF. Then the molecules can diffuse into the brain tissue or will be cleared by the CSF flow into the lymphatic vessels and susequently into the systemic circulation.

Factors Affecting Nasal Drug Delivery to the Brain

The size of the molecule is the major determinant in whether a substance will be absorbed across the nasal respiratory epithelium and/or transported along the olfactory pathway. Fisher *et al*. demonstrated an almost linear relationship between the log (molecular weight) and the log (% drug absorbed) of water-soluble compounds (190-70,000 Da)[17,18]. In general, molecules weighing more than 1000 Da are absorbed far less efficiently than smaller molecules[19]. However, the bioavailability of larger molecules may be increased with the use of permeation enhancers.

Other factors affecting delivery to the brain include the degree of dissocation (determined by the pK_a of a substance and the pH in the surrounding area)[20], and lipophilicity (higher lipophilicity results in better transport)[21]. Once a drug is in the brain, it can be further influenced by BBB efflux transporter systems like P-glycoprotein (P-gp)[22]. Graff and Pollack (2003), however, found that uptake into the brain was enhanced when drugs were administered in combination with the P-gp efflux inhibitor, rifampin.

Nose-to-Brain Research

Researching nose-to-brain transfer of drugs in humans must, for obvious reasons, either employ indirect visualization of drug transfer (e.g., effects on event-related-potentials), measurement of drug concentrations in the CSF during surgery, or simple monitoring of CNS effects. Such studies have clearly indicated that drugs can be delivered to the brain in this manner, but they give no clear-cut evidence regarding the role of transfer. Because of this limitation, studies of the olfactory pathway as a conduit for transmission of drugs to the CNS have mostly made use of animals having substantially different ratios of olfactory-to-respiratory epithelium than humans. However, the mechanisms of transfer remain the same and are worthy of thorough investigation. To date, more than 50 drugs and drug-related compounds have been reported to reach the CNS after nasal administration in different species.

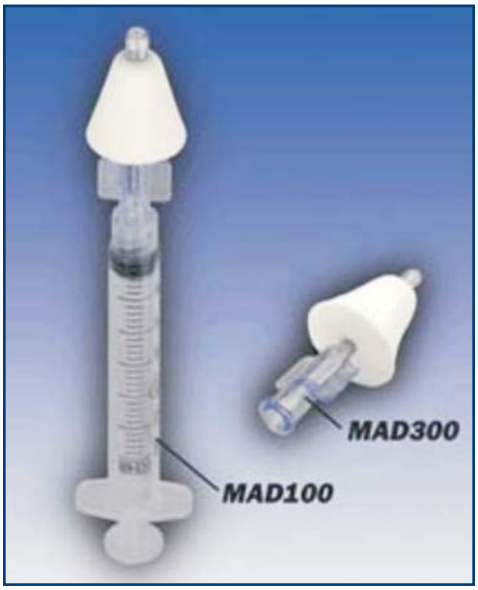

Figure 2. *MAD® Nasal drug delivery device by Wolfe Tory Medical, Inc. (http://www.wolfetory.com/nasal.html)*

A growing number of recent reports have demonstrated the effectiveness of intranasal administration of neuroprotective agents in decreasing ischemic brain injury. For example, Ying *et al*. (2007) recently reported that intranasal administration of NAD^+ profoundly decreased brain injury in a rat model of transient focal ischemia[23]. Similarly, Wei *et al*. (2007) showed that intranasal administration of the PARG inhibitor gallotannin decreased ishemic brain injury in rats[24]. Such agents are believed to provide neuroprotection by diminishing or abolishing activation of poly(ADP-ribose) polymerase-1 (PARP-1), which plays a significant role in ishemic brain damage. NAD^+ was observed to reduce infarct formation by up to 86% even when administered at 2 hours after ischemic onset. Because PARP activation appears to be a downstream ischemic event, it may be worthwhile to also investigate the ability of IN administration of agents such as antiporters or NMDA receptor blockers to provide neuroprotection against the more upstream events of global ischemia such as membrane depolarization and excitotoxicity.

Applicability to Cryonics

IN administration of neuroprotective agents would appear to be a possible way to circumvent many of the problems encountered when attempting to administer neuroprotective agents during a typical cryonics case. No special training is required to administer drugs to the nasal cavity – IN administration is easy enough even for relatively unskilled stabilization team members and does not require sterile technique. Indeed, using a simple instrument such as the MAD® Nasal drug delivery device (Fig. 2) provides the possibility of self-administration of neuroprotective and cardiovascular agents in emergency situations (e.g., at the first sign of cardiac arrest). Consequently, time-sensitive ischemia medications can be administered and distributed throughout the brain more quickly and, importantly, can provide protection to the brain before cooling is sufficient to prevent major ischemic damage.

There is also evidence indicating that many agents active in the CNS are more effective when given nasally than when given by other routes. This suggests that smaller doses may be used when bypassing the BBB in this manner, allowing for the possibility of rapid administration of neuroprotective agents that work in low dosages immediately after cardiac arrest.

Limitations on the use of intranasal delivery as a means to bypass the BBB still exist, including limitation of the concentrations achievable in different regions of the brain, which will vary with each agent. Indeed, data regarding IN transport rates and bioavailability of specific drugs are still scarce. However, the advantages of IN administration appear considerable and worth further investigation to determine the extent to which they may benefit cryonics. ■

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THE ROAD LESS TRAVELED: ALTERNATIVES TO CRYONICS

A VERY PRELIMINARY SURVEY

By R. Michael Perry, Ph.D.

Introduction

A man lies critically injured on the highway, one leg pinned under his battered Harley-Davidson. He had just passed a car while approaching an intersection, but he couldn't quite make it around the pickup stopped in the road just ahead. The man is alive but unconscious; blood dribbles from his mouth as his faltering, shallow breathing is assisted by a paramedic who has just arrived on the scene. Forty-five minutes later the man expires at a nearby hospital, the victim of a brain hemorrhage. The brain, which appears during the mandatory autopsy to be largely undamaged despite the fatal trauma, is placed in a fixative solution after some delay at mostly refrigerated temperature…

The above, true-life scenario played itself out last September for a relative of mine who had expressed interest in cryonics but not completed any arrangements. He had, however, signed a "Declaration of Intent" expressing a wish to be cryopreserved. Unfortunately, with only that single signed document in place, the usual cryonics procedures could not be applied and the best that could be attempted was a "salvage job." The brain, as I write this, still rests in a refrigerated (liquid) fixative bath where it is undergoing a slow diffusion of cryoprotectant, after which it will be cooled and placed in liquid nitrogen for long-term care.

Tragic events involving persons who are interested in cryonics but who have not signed up furnish the type of case where an alternative to the usual procedures is needed, at least for the all-important preliminary stages. This presumes that funding can be arranged as needed for the long-term, cryogenic care. However, there are also many cases where there simply is not enough funding, and something entirely apart from the usual expense of cryonics is desired.

The reasons for considering an alternative to cryonics includes the potential for much easier, cheaper long-term care and thus greater security against future contingencies such as social unrest, energy and materials shortages, or economic hardships affecting the cryonics provider. Doubts have been expressed that alternative procedures would be adequate for the goal of eventual revival of the cryonics patient, given the exacting requirements that must be met versus the state of the art in current fixation technology. But this is not enough reason to give up. A thorough study of existing fixation methods, such as that used in my relative's case, is called for, along with attempts to develop better methods.

What follows is a very preliminary report. I have looked into various possible fixative techniques and their effects. Much further work is called for, both in surveying existing research and, eventually, in pursuing new research. Of what I have been able to study so far, some approaches show interesting success. As an overall impression: tissue ultrastructure, which is thought to be critical to the problem of revival (particularly for brain tissue), appears to be well-preserved by a number of different methods that start with aldehyde fixatives such as formaldehyde and glutaraldehyde. Some difficulties with fine-scale preservation have been noted. But these methods, I think, strongly merit further investigation and, in some cases at least, provisional use by cryonics providers when standard cryonics procedures are unavailable and the only alternative is to decline the case.

Philosophical Issues

A basic question in cryonics is whether preservation is good enough to offer a reasonable prospect of eventually restoring a preserved patient to full health and vigor. At minimum there should be enough structural preservation so that the healthy state of the patient can be inferred from the remains. If the healthy state can be known then it should be possible, using future technology, to make appropriate repairs to bring about the desired recovery so the patient will return to consciousness and full functionality. The repairs could be extensive, so if, for example, only the brain was preserved the entire rest of the body would need to be replaced. Repairs to the brain, too, could be substantial. The issue will arise, for some more acutely than for others, of whether the resulting patient, even if similar to the original in every respect, really would "be" that original person or, perhaps, just a faithful copy.

While I think there are reassuring answers for issues such as these, the subject is beyond the scope of this study. Instead, I will be mainly concerned with the ability of the various preservation techniques to achieve quality structural preservation. My "gold standard" will be based on an information-theoretic criterion: The healthy brain should be inferable from the state or condition of what is preserved—this I sometimes refer to as a *favorable* preservation.[1] In practice it is presently unknown whether *any* preservation should be considered favorable in this sense, including standard cryopreservations. But we can at least look at the results and see, in the case of brain tissue, whether basic structures such as neuron cell bodies, axons, dendrites, and synapses seem well-preserved (See Figures 1 & 2 on page 23).

I think, too, that with our present ignorance of the limits both of preservative methods and of future resuscitation technology, together with philosophical uncertainties, we should not be too hasty in dismissing any preservative technology. Instead we should be willing to grant some benefit of doubt. Imperfect or unfavorable preservation could result

both from the methods used and from the condition of the patient at the time a procedure is started. But even cases where significant information loss occurs the results could be worth the effort. We must keep in mind that future repair technology, though it too will have limitations, will be powerful in ways hard to appreciate today. Debilities other than amnesia should be fully curable, for example, while even amnesia could be mitigated through use of historical records or in other ways.

On this ground, then, I favor a "no patient left behind" policy in which preservation is attempted even when certain, expensive methods are out of reach and some damage has occurred such as through autopsy or warm ischemia.[2] (My relative here is an obvious case in point.) The brain, as the seat of the personality, is the primary focus of this article, although some interesting studies of other tissues also show details at the cellular level, particularly some work with ancient fossils.

Unintentional and Contingency Preservation

The world of nature may seem an unlikely place to look for high-quality, long-term preservation of biological tissues, yet something of this sort does occur, relatively speaking, in such remains as natural frozen mummies and specimens embedded in amber. Here I include a very brief summary of some of these unintentional preservations, then consider a more recent, intentional but "contingency" preservation in which fixation was applied only after long delay. These cases are important, if for no other reason than because they show that surprising amounts of fine structure can be preserved under adverse circumstances, suggesting that the goal of favorable preservation may be reachable through a variety of approaches, not necessarily cryogenic.

Tyrolean Ice Man. The 5,200-year-old Tyrolean Ice Man discovered in a glacier in 1991 is the oldest known frozen human mummy. Although frozen at high subzero-Celsius (non-cryogenic) temperature for most of the five millennia since his death, it is clear that some thawing and refreezing occurred, including an incident immediately following discovery. Despite the adverse circumstances, a 1998 study reports interesting findings for many parts of the anatomy, in which many details at the cellular level can be seen, albeit with substantial loss. In the brain there was preservation of the myelin sheaths of axons, even though the material of the axons and the neuron cell bodies were mostly obliterated.[3] Details of the myelin sheaths were discernible down to 10 nanometers, or about 65 carbon atom-diameters.

Peat Bog Burials. In addition to glaciers, peat bogs have furnished surprising examples of preservation. One such location, a swampy pond near Windover, Florida, was excavated in the 1980s. Remains of several individuals with recognizable if greatly shrunken brains were found. While there was much damage, definite cellular remnants could be seen on light and electron microscopic examination: axons; cerebellar purkinje cells in approximately their original spatial configurations and what appeared to be remnants of neurons. DNA was recovered, as well. The estimated age of this material was 7,000-8,000 years. The preservation, limited though it was, is all the more striking since it involved above-freezing storage under water, which normally results in rapid decomposition. Apparently the lack of oxygen and neutral pH in the water-soaked peat greatly slowed the normal processes of breakdown, leaving us this tantalizing, if fleeting, glimpse of ancient humans.[4]

An excavation of a Florida peat bog, similar to that shown, in the 1980s recovered several individuals, believed to have died 7,000-8,000 years ago. Apparently the lack of oxygen and neutral pH in the water-soaked peat greatly slowed the normal processes of breakdown, leaving behind a fleeting glimpse of ancient humans.[4]

Amber Fossils. Turning to something much more ancient still, study was made in 2005 of a cypress twig preserved in Baltic amber for 45 million years. As reported in the *Proceedings of the Royal Society,* the "Transmission electron micrographs revealed highly preserved fine structures of cell walls, membranes and organelles" with cells more or less normal-appearing. Earlier studies of amber-preserved material also showed fine details of cell structure in both animal and plant tissues. The new study used a novel, resin-embedding technique to prevent breakup of the extremely fragile ancient tissue under microtome sectioning, allowing full cross sections to be prepared for microscopic examination. DNA and RNA were not detected but hopes were raised that these species-specific macromolecules were present at least in inferable form and might be deciphered with suitable techniques.[5]

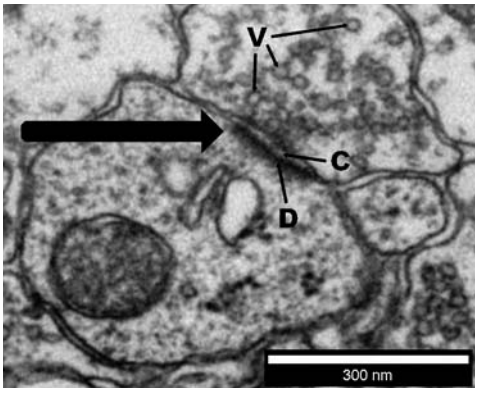
A Recent Case. I conclude this section with a case of recent, intentional preservation that occurred under adverse circumstances. The body of a woman was kept in a mortuary for 2 months at 3°C with no fixation, then the brain was removed and fixed in a phosphate-buffered formaldehyde solution (4.5%) for 9 weeks. Subsequent examination of the brain showed macroscopically visible surface deterioration; histologically, however, normal brain structures were preserved including all important cell types (neurons, astrocytes, oligodendrocytes, microglia), neuropil, axons, and myelin sheaths. From a cryonics standpoint the preservation was not good but, I would say, not negligible either.[6]

Basic Fixation: Theory, Materials, and Methods[7]

When a patient undergoes cardiac arrest, blood no longer circulates and cell metabolism is compromised. Without special intervention, deterioration of the tissues soon sets in. Cryonics protocols are designed to halt this deterioration by placing the patient's tissues in a condition of *biostasis*, in which biological and chemical activity is halted. More specifically, biostasis (1) halts any biological activity within the tissues, (2) protects the tissues from attack by microorganisms such as bacteria, and (3) stabilizes the tissues so that long-term care in an unchanging state is practical. Biostasis in this case is achieved through use of low (cryogenic) temperature, –100°C or lower.

An acceptable substitute for a cryonics protocol, a non-cryogenic fixation procedure, must likewise accomplish the goals of biostasis and permit long-term care of the patient or the patient's brain in an unchanging state. There are several possibilities.

Cross-linking fixatives use covalent chemical bonds to tie down the normally reactive molecular components such as proteins and peptides that make up tissues. The most widely used fixative, formaldehyde, is of this type, as is another popular fixative, glutaraldehyde. The two are sometimes used in combination to achieve what is thought to be a better result than either alone would accomplish. Formaldehyde has one aldehyde group per molecule to accomplish the crosslinking, whereas glutaraldehyde has two and also a longer molecule and thus is able to bond more securely by stretching its bonding over greater distances. However, the extra power of glutaraldehyde comes at a cost: its



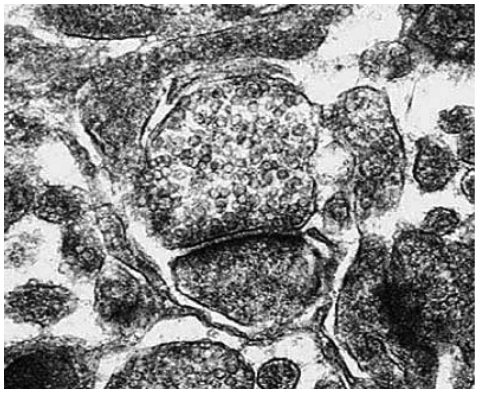


Figure 1 (top) & Figure 2 (bottom): *Figure 1 (courtesy of Narayanan Kasthuri and Kenneth Hayworth) shows a 38-nanometer-thick tissue slice of aldehyde- and osmium-fixed mouse brain cortex embedded in epon resin and sectioned and collected on Harvard's Automatic Tape Collecting Lathe Ultramicrotome (ATLUM). This scanning electron microscope (SEM) contrast-reversed image shows synapse (large arrow), synaptic vesicles (V), synaptic cleft (C) separating presynaptic and postsynaptic membranes, and postsynaptic density (D). Brain ultrastructure, including synapses which are believed important for encoding memory, appears to be well-preserved. Figure 2 (courtesy of 21st Century Medicine), showing a SEM image of a rabbit brain synapse perfused with M22 for 60 minutes, shows comparable ultrastructure preservation.*

molecules are larger and thus do not penetrate into tissue as fast. Using it in combination with formaldehyde means getting what is actually a good fixative, formaldehyde, in fast while the even-better fixative, glutaraldehyde, can move in at a more leisurely pace and make its extra contribution. As one case in point, my relative's brain was preserved in a solution of 2% formaldehyde, 4% glutaraldehyde.

Formaldehyde is the most widely used fixative for the various requirements outside of cryonics; it and glutaraldehyde have also been most used in cryonics when called for by special circumstances. Other fixatives have more specialized uses, which still may have importance. *Precipitating fixatives* such as acetone, ethanol, methanol, and acetic acid achieve their effects by reducing the solubility of the proteins that largely comprise the tissues. *Oxidizing fixatives* use oxidation to crosslink proteins; osmium tetroxide is one fixative in this class that has uses connected with high-magnification microscopy.

Fixation of a tissue sample requires a reliable method of delivery. With a whole organ such as the brain it may be possible to use perfusion or pumping the fixative solution into the organ through the vascular system. The alternative is to immerse the sample in a fixative bath, during which the fixative penetrates the tissue more slowly through simple diffusion. For my relative, diffusion was used since the brain's vascular system had been traumatized; probably several days of diffusion was adequate for nearly complete penetration under refrigerated, above-freezing conditions.

When the sample—a brain, say—is fixed, we must ask (1) whether the preservation of detail is adequate and (2) whether the sample will remain stably fixed long enough to accomplish the desired purpose. These are demanding requirements for alternative procedures. Since decades or centuries may be needed to develop technology to attempt resuscitation, long-term stability becomes an especially important issue. As with cryonics itself, the basic answers are unknown. Some encouragement is provided by the high level of detail seen in preserved brain samples using, for example, formaldehyde fixation. Ultrastructural details under the high magnification of electron microscopy (10,000x plus) are quite clear, though this alone is not a demonstration that all the details one would like are present. However, the same problem exists with tissue preserved cryogenically—the answer to whether the preservation captures fine enough details is unknown though there are at least some encouraging signs along with reasons for concern.

To return to the problem of long-term stability, one consideration is whether the fixative is uniformly distributed throughout the sample. Preservation could be compromised if there are "islands" of tissue that were not properly fixed. (A somewhat analogous, though possibly less serious, problem occurs with cryopreservation. If cryoprotectant is not uniformly distributed in the tissue prior to lowering the temperature to the cryogenic range there could be freezing damage in the "islands" of tissue not protected, even though the cold penetrates uniformly everywhere and will prevent deterioration.) So far we have considered "wet" preparations only, in which the sample is to be kept immersed in a liquid bath. Water molecules in particular are small and move rapidly, and their constant, reaction-prone hammering in, say, a specimen preserved in an aqueous aldehyde solution could have unwanted long-term effects. For greater stability and durability it would thus be desirable to have some form of dry preparation for long-term care; this will now be considered.

Dry Preparations for Biostatic Care

A number of techniques, some dating back more than a century, have been developed for dry storage of preserved biological specimens. One motivation for developing these techniques has been to provide specimens, such as whole, human organs, as a teaching aid. The aim here is to render the organ in a lifelike but inert and touchable form for macroscopic or gross anatomical study or exhibits. One is not concerned with microscopic details but only those features visible to the unaided eye.

Another, very different application, for which different embedding methods have been developed, is to render specimens in a form that can be cut into thin sections for microscopic study. Here the focus is on capturing fine structure but not with preserving the whole mass intact. In cryonics, of course, we are interested in both the fine structure and in preserving the whole specimen intact, though not for either gross anatomical study or microscopic study requiring fine slicing. These differences need to be borne in mind in the short survey that follows. The techniques discussed next look promising in some cases but could be improved for our purpose.

Desiccation.[8] Nature has pioneered various forms of preservation through desiccation. In addition to amber fossils (normally quite dehydrated) natural mummies found in desert environments capture such detail as DNA and large anatomical features, though brain tissue is poorly preserved. For scientific purposes, desiccation in the form of freeze-drying has been used to preserve anatomical specimens in a lifelike, inert form for study or exhibits. The specimen is first frozen, then allowed to dry out at below-freezing temperature, generally under high vacuum to accelerate the process. As the ice evaporates, the structural shapes remain largely unchanged, even though the final result is delicate and will need protection from oxidation and moisture. Human brain tissue in large sections (order of 200g or about 15% of a whole brain) has been freeze-dried after formaldehyde fixation and shows some structural details such as capillaries under the scanning electron microscope, though much damage has occurred. Some of the damage resulted from freezing itself and might be mitigated through use of cryoprotectants; as usual, the potential of this process is unknown.

Early Embedding Techniques.[9] More than a century ago paraffin embedding was used to preserve biological specimens in a form that would be amenable to fine sectioning for microscopic study, and it is still used. In one procedure from a 1902 reference, small tissue samples fixed in formaldehyde and dehydrated with ethanol are embedded in melted paraffin, a white or colorless, tasteless, odorless, water-insoluble, solid substance not easily acted upon by reagents, which reaches a temperature of 55°C (131°F). The melt is rapidly cooled to reduce crystallization and improve transparency in the resulting solid embedding. A related technique in the same reference uses a plastic-like material, celloidin, in place of paraffin. This permits embedding at room temperature through use of an ether-ethanol solvent; it is more suited to larger specimens, though also requiring full dehydration.

Plastination. An embedding technique developed by Gunther von Hagens in Germany in the 1970s,[10] plastination starts with a previously fixed biological specimen in an aqueous medium. Water and fats (lipids) are then replaced with a liquid plastic resin monomer, which is hardened or cured by polymerization. The resulting, resin-impregnated specimen is dry, odorless, and durable. Silicone resin yields a flexible or rubbery specimen suitable for macroscopic study or exhibits; epoxy resin can be used to produce thin, rigid sections suitable for microscopic study. Silicone impregnation has also been adapted to microscopic study, with good results.[11] Other desired effects are possible using different polymers,[12] and substantial further adaptations might be feasible.

A possible drawback of this approach, from the standpoint of preserving the fine structures that are especially important from a cryonics standpoint, is the relatively harsh regimen needed to produce the finished product. Typically, the process starts with an aldehyde-fixed specimen in aqueous solution. The specimen is placed in acetone, and successive changes of the bath remove water and fats. Finally the resin monomer is introduced, the remaining acetone is removed by vacuum, and induced catalysis yields the desired polymerization. Concerns have been raised about whether defatting would obliterate important brain information, though there does not appear to be strong evidence of this. (Here it is appropriate to mention that lipids nevertheless could contain important information; preservation of lipids is a difficult process that has not been covered in this preliminary survey but deserves consideration.)

Polyethylene Glycol.[13] "Polyethylene glycol" (PEG) is actually a family of water-soluble polymers of ethylene glycol in which the molecules form long, linear chains. A typical batch will contain a mixture or distribution of polymer chains of varying lengths. Longer chains result in a PEG with a higher melting point, which is a waxy solid at room temperature or below. Its water solubility and other characteristics make it possible to introduce PEG into tissue without using dehydration, defatting, heating, or additional polymerization, suggesting that PEG could overcome possible problems encountered with many other forms of embedding. PEG, however, does not seem much used for biological embedding and may not be as satisfactory as other methods even for the special requirements that relate to cryonics. As usual, there are important unknowns here and further investigation is called for.

Discussion and Conclusions

The agonizing question will continually arise of what to do in an emergency where cryonics is desired but arrangements are not in place. An alternative to just giving up is to consider some non-cryonics procedures, such as chemical preservation of the brain, as a temporary solution until cryopreservation can be arranged or, if this eventuality is not feasible, as a long-term option itself.

In such a case, the question arises of whether the preservation is adequate to meet the unknown, presumably demanding requirements if the patient is to be repaired without deficits by future technology. Revival in such a "perfect" form will happen only if enough identity-critical structure is present in some inferable form. The high degree of structural preservation seen in some fixation methods raises hopes that in fact such a degree of preservation is achievable through non-cryogenic means.

In addition to the (by appearance) encouraging results obtainable with water-based fixatives in a liquid bath, there is the prospect of embedding the specimen (normally the patient's brain) in a solid matrix for long-term care. Certainly there are unknowns with such an approach but one finds this in the standard cryonics procedures too.

The alternatives discussed herein offer the additional advantage of more security per unit cost for conditions of social unrest or economic hardship which may occur over the long interval before revival will be attempted. In addition, there is reason to consider preservation methods that knowingly fall short of the best methods currently possible, when, for example, the cost of better treatment is prohibitive. The alternatives must be taken seriously, then, for a number of reasons, and more work is needed to both assess and enhance their potential. Along with this, so long as invalidating difficulties are not discovered, it is imperative that such alternatives become more readily available—through some appropriate organization—and widely advertised. ■

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WHAT IS CRYONICS?

Cryonics is an attempt to preserve and protect the gift of human life, not reverse death. It is the speculative practice of using extreme cold to preserve the life of a person who can no longer be supported by today's medicine. Will future medicine, including mature nanotechnology, have the ability to heal at the cellular and molecular levels? Can cryonics successfully carry the cryopreserved person forward through time, for however many decades or centuries might be necessary, until the cryopreservation process can be reversed and the person restored to full health? While cryonics may sound like science fiction, there is a basis for it in real science. The complete scientific story of cryonics is seldom told in media reports, leaving cryonics widely misunderstood. We invite you to reach your own conclusions.

HOW DO I FIND OUT MORE?

The Alcor Life Extension Foundation is the world leader in cryonics research and technology. Alcor is a non-profit organization located in Scottsdale, Arizona, founded in 1972. Our website is one of the best sources of detailed introductory information about Alcor and cryopreservation (www.alcor.org). We also invite you to request our **FREE** information package on the "Free Information" section of our website. It includes:

- A 30-minute DVD documentary "The Limitless Future"
- A fully illustrated color brochure
- A sample of our magazine
- An application for membership and brochure explaining how to join
- And more!

Your free package should arrive in 1-2 weeks.

(The complete package will be sent free in the U.S., Canada, and the United Kingdom.)

HOW DO I ENROLL?

Signing up for a cryopreservation is easy!

Step 1: Fill out an application and submit it with your $150 application fee.
Step 2: You will then be sent a set of contracts to review and sign.
Step 3: Fund your cryopreservation. While most people use life insurance to fund their cryopreservation, other forms of prepayment are also accepted. Alcor's Membership Coordinator can provide you with a list of insurance agents familiar with satisfying Alcor's current funding requirements.
Finally: After enrolling, you will wear emergency alert tags or carry a special card in your wallet. This is your confirmation that Alcor will respond immediately to an emergency call on your behalf.

Call toll-free today to start your application:
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www.alcor.org

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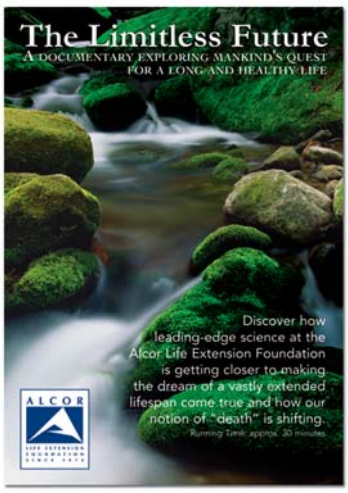

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