18. Cryoprotection

Cryoprotective perfusion is the most crucial phase of human cryopreservation procedures. If cryoprotective perfusion is compromised, freezing will produce extensive damage to the fine structure of the brain of the patient, regardless of the quality of the cryonics organization's standby and stabilization efforts. Since its inception, most sensible advocates of cryonics have, therefore, always recommended some form of cryoprotection to mitigate ice formation during storage a low temperature. Since the year 2000, the Alcor Life Extension Foundation introduced vitrification as an alternative to conventional cryopreservation with the aim of abolishing freezing altogether. In this section, we will discuss aims of cryoprotective perfusion, the history of its technologies and protocols at Alcor, and special applications such as whole body cryopreservation or field cryoprotection.

Objective

The objective of cryoprotective perfusion is to replace blood and the liquid parts of cells with a solution of cryoprotectant agents (CPAs) to reduce or, ideally, eliminate freezing damage when the body or brain is cooled below 0 degrees Celsius. To this purpose vascular access is obtained and perfusion technologies are used to introduce the CPA solution of choice such that the concentration of CPA ingredients increases with time while the temperature of the patient is reduced. Measurements at the venous side of the patient are used to determine when the target concentration of the CPA solution has been reached inside the body.

After completion of cryoprotectant perfusion the patient is gradually cooled below the glass transition temperature (Tg) for long term care. While some patients at Alcor are maintained at a temperature between Tg and liquid nitrogen, the great majority of patients at Alcor and other cryonics storage

facilities have been immersed in liquid nitrogen at a temperature of –196 degrees Celsius.

See Section 20 for more information about the maintenance of cryopatients.

Cryoprotectant Solutions

Cryoprotectant solutions contain a variety of ingredients that serve different purposes. All cryoprotectant solutions contain a particular set of ingredients that together comprise a carrier solution, also called a vehicle solution. Carrier solution ingredients are not considered to be cryoprotectants. Most carrier solution ingredients remain at constant concentration while the concentration of cryoprotectant ingredients (CPAs) in the solution changes during a cryopreservation process.

The carrier solution ingredients are all "non-penetrating," which means that they don't penetrate cell membranes because they are either large molecules or ionically charged small molecules. Carrier solution ingredients include pH buffers (usually adjusted to make a solution pH of 8) and osmotic agents to give the solution a tonicity near 300 milliOsmolal (excluding CPAs) so that cells don't osmotically swell to larger than their normal volume during a cryoproreservation process. The carrier solution may also contain small concentrations of calcium and magnesium ions to stabilize cell membranes. However the concentration of calcium and magnesium is lowered as CPA concentration increases. Otherwise these ions tend to precipitate in the presence of high concentrations of CPA ingredients.

CPAs (cryoprotective agents) are the ingredients of a cryoprotectant solution that are specifically included to inhibit ice formation. During circulation of cryoprotectant solution through blood vessels (perfusion), the concentration of CPA ingredients is increased until a desired target arterial concentration is reached.

There are two types of CPAs, penetrating and non-penetrating.

Penetrating CPAs are small uncharged polar molecules, typically of molecular weight less than 90, that are small enough to penetrate cell membranes (typically through aquaporin channels) on a timescale of minutes.

When exposed to penetrating CPAs, cells initially shrink as water rushes out of the cell in response to the osmotic stress of the CPA outside the cell. Over several minutes, intracellular and extracellular concentrations of CPA equalize by diffusion, and the cell returns to normal volume (as dictated by the tonicity of the carrier solution). During perfusion, cell dehydration in response to increasing CPA concentration manifests as dilation of blood vessels, causing a decrease in vascular resistance, causing an increase in solution flow if perfusion pressure is held constant. This flow increase is most likely to be seen at the beginning of a cryoprotectant perfusion ramp ("ramp" means planned increase in CPA concentration over time), when even a small change in CPA concentration causes a large proportional change in extracellular solution tonicity.

Non-penetrating CPAs are large molecules, typically polymers, that are too large to penetrate into cell interiors. By penetrating through capillary gap junctions (but not cells), non-penetrating CPAs provide extra protection against ice formation in the extracellular space of tissue. This extra protection is needed because ice has a greater tendency to nucleate (initially form) outside cells than inside cells, and cytoplasm inside cells has its own natural polymers that in combination with penetrating CPAs help protect against ice formation.

For more information about cryoprotectants, how cryoprotectant technology has advanced, and the difference between freezing and vitrification, see the article "How Cryoprotectants Work" in *Cryonics* magazine, 3rd Quarter 2007, archived on the Alcor web site.

Cryoprotectants at Alcor

Since its inception Alcor has been guided by theoretical and practical cryobiology research to incorporate the most promising developments in mitigating ice formation. Initially DMSO was Alcor's cryoprotectant of choice, reflecting the general popularity of DMSO as a cryoprotectant in the 1960s and its (reported) superior ability to penetrate cells. For example, 5% of DMSO, followed by 20% DMSO in modified Collins solution, was used to perfuse Frederick Chamberlain, Jr. (Alcor's first neuropatient) in 1976.

Around 1977, following an extensive correspondence between Michael Darwin and Jerry Leaf, Alcor decided to switch from the use of DMSO to glycerol based on several observations that even low concentrations of DMSO increased whole body edema in cryonics patients. Concentrations of glycerol were chosen to satisfy the so-called Smith Criterion, i.e. Audrey Smith's discovery that golden hamsters could survive 60% of the water in their brains being converted into ice with no ill effects. In 1992, a consulting cryobiologist recommended to take the glycerol concentration as high as possible. Since glycerol has a high viscosity at low temperatures, in practice this recommendation translated in a target concentration range between 7.5M and 8.0M, which in combination of the glycerol-induced dehydration of the brain inhibited most, but not all, ice formation.

From the year 2000 Alcor announced switching from high molarity glycerol to a new generation of vitrification agents designed by the cryobiology company 21st Century Medicine, modelled after its vitrification agent VM3 (see Section 1). The first two agents were named B1C and B2C. B1C and B2C were the first "hyperstable" vitrification agents used at Alcor and formulated to inhibit ice formation at relatively low cooling rates (~ 0.1 degree C per minute). B1C was only used in a few of cases and was shortly replaced by B2C which increased the concentration of penetrating components relative to polymers to reduce viscosity. Due to concerns about whole body edema and the need to develop new equipment for cooling whole bodies at the faster rates preferred for vitrification, B2C was only available for neuro-preservation cases.

In 2005 Alcor introduced 21st Century Medicine's low toxicity vitrification solution M22, thereby closing the gap between the state of the art in mainstream cryobiology for the vitrification of complex mammalian organs, and cryoprotectants used in cryonics. M22 was Alcor's first solution with backing in the peer reviewed literature for recovering kidneys from –45 degrees C and good ultrastructural brain vitrification. Additives also allowed whole body perfusion, which made vitrification available for all Alcor members who had elected whole-body preservation as opposed to neuropreservation. See Table 18-1 for a history of Alcor cryoprotectants.

Year	Cryoprotectant
1976	20% DMSO
1980	3.0M Glycerol
1987	4.5M Glycerol
1992	8.0M Glycerol
2001	B1C (neuro only)
2001	B2C (neuro only)
2005	M22 (separate neuro and whole body formulations)

Table 18-1. History of cryoprotectants used at the Alcor Life Extension foundation.

M22 Cryoprotectant

To understand cryoprotectant design, we reproduce the formula of M22 in Table 18-2 and will discuss its individual components and design principles.

Dimethyl sulfoxide	22.305% w/v
Formamide	12.858%
Ethylene glycol	16.837%
N-methylformamide	3%
3-methoxy-1,2-propanediol	4%
Polyvinyl pyrrolidone K12	2.8%
X-1000 ice blocker	1%
Z-1000 ice blocker	2%

Table 18-2. Composition of M22 cryoprotectant solution at full strength, also called "1x" concentration or CNV (concentration necessary to vitrify). During

cryoprotective perfusion, the concentration of M22 ingredients increases from zero to full strength. Not shown are vehicle solution (aka carrier solution) ingredients that remain at constant concentration during perfusion.

The name of M22 refers to –22 degrees C, which is the temperature at which the full-strength solution is perfused into kidneys in published organ cryopreservation experiments. The core components of M22 are are the penetrating cryoprotectants DMSO, formamide, and ethylene glycol. The equimolar DMSO and formamide combination reflects cryobiologist Gregory Fahy's discovery that DMSO can neutralize the toxicity of formamide. The weak glass former ethylene glycol is added to this essential non-toxic core of DMSO and formamide to further increase cryoprotectant concentration. While methylated cryoprotectants like n-methylformamide and 3-methoxy-1,2-propanediol exacerbate cryoprotectant toxicity in high concentrations, in small concentrations they can improve the solution's resistance against ice formation and reduce viscosity. Addition of the non-penetrating cryoprotectant PVP K12 reflects the fact the intracellular environment of the cell is more resistant to freezing than the extracellular environment, and thus can tolerate a slightly lower overall cryoprotectant concentration. X-1000 (a co-polymer of polyvinyl alcohol) and Z-1000 (polyglycerol) are 21st Century Medicine's propriety "ice blockers" which reduce the concentration necessary to vitrify and critical cooling rate by inhibiting ice nucleation generally (X-1000) and ice nucleation due to protein contamination specifically (Z-1000).

The vehicle solution for M22 is called LM5 to reflect the 50% reduction of glucose (as compared to the older vehicle solution RPS-2) in favor of equimolar concentrations of lactose and mannitol, to solve compatibility problems with the ice blockers. LM5 works together with the non-penetrating CPA components to create an overall hypertonic solution that is effective in mitigating "chilling injury." At Alcor a minor adjustment of LM5 named B1 is used for cryopreservation, which includes an additive to inhibit swelling during cryoprotective perfusion. B1 is also the washout solution that precedes the start of cryoprotectant perfusion. See Table 18-3.

The initial stage of cryoprotective perfusion is conducted with an arterial perfusate temperature close to +3 degrees Celsius. When the concentration of

M22 ingredients reaches 50% of CNV (Concentration Necessary to Vitrify),as measured by the venous refractive index, the temperature is dropped to about –3 degrees Celsius and the concentration of M22 ingredients is quickly ramped up to 100%.

Chemical	MW	Molar Conc.	Grams/Liter
Glucose	180.16	90 mM	16.214
Mannitol	182.17	45 mM	8.198
Alpha-Lactose Monohydrate	360.31	45 mM	16.214
Potassium Chloride	74.55	28.2 mM	2.102
Potassium phosphate dibasic trihydrate	228.22	7.2 mM	1.643
Gluthathione (reduced)	307.32	5 mM	1.537
Adenine HCl	171.59	1 mM	0.172
Sodium Bicarbonate	84.01	10 mM	0.840
Calcium Chloride Dihydrate			0.147
10% w/v	147.01	1.0 mM	1.47 ml
Magnesium Chloride Hexahydrate			0.407
20% w/v	203.3	2.0 mM	2.035 ml
Proprietary Additive			

Table 18-3. Components of B1 vehicle solution used at Alcor. B1 consists of the LM5 vehicle solution developed by 21st Century Medicine, Inc., for kidney cryopreservation plus a proprietary additive. The concentration of the LM5 vehicle solution ingredients doesn't change as the concentration of the M22 ingredients of Table 2 increases during cryoprotective perfusion.

Cryoprotectants and the Brain

The fact that perfusion of the brain with cryoprotectants causes the brain to physically shrink has been known in cryonics since cryonics organizations started using burr hole observations to evaluate the effects of cryoprotectant perfusion. This shrinkage is often called dehydration, although technically all tissue treated with cryoprotectants dehydrates (loses water) whether it shrinks or not. The cause of this shrinkage is that most cryoprotectants have poor blood brain barrier (BBB) permeability. The usual cryobiological "shrink

swell" response of tissue in response to CPA exposure as water first leaves tissue by osmosis (shrink) and is then replaced by CPAs by diffusion (swell) is mostly just "shrink." Glycerol in particular has been associated with severe brain dehydration (except in canines, the brains of which appear to be anomalously permeable to CPAs when pets are cryopreserved). Most of the penetrating cryoprotectants in M22 have relatively slow BBB penetration. PVP K12 and the ice blocker ingredients of M22 are presumed to not penetrate the BBB at all.

HPLC studies (unpublished) do show significant penetration of the low molecular weight CPA components of M22 into the brain, but not at a rate sufficient to prevent brain shrinkage during perfusion. Since there are no open capillary gap junctions in the brain (the definition of the blood brain barrier), penetration of CPAs into the brain is presumed to occur directly through capillary endothelial cells, with cryoprotectants entering endothelium cells on the intravascular side and exiting on the extravascular side following diffusion gradients from high to low concentration.

The inability of the non-penetrating CPAs to leave brain blood vessels, and limited penetration of other CPAs into the brain, should not be interpreted to mean that the brain isn't cryoprotected by perfusing cryoprotectant solutions through it. Water moves readily across the BBB in response to "water activity" gradients (osmosis). The temperature at which ice would melt in a solution is one way of measuring water activity. When a solution with a low melting point, such as M22 with a melting point of -55 degrees C, is perfused through brain blood vessels, water is drawn from brain tissue into the blood vessels following the gradient of high water activity in the brain to low water activity in the vessels. Water will continue to leave tissue and increase the concentration of solutes in tissue until at equilibrium the water remaining in brain tissue has the same low melting point as the solution in the vessels.

This loss of water and resulting melting point depression makes brain tissue more resistant to ice formation. Differential scanning calorimetry (DSC) study of tissue samples from brains perfused with M22, and microscopy studies of whole brains cryopreserved after perfusion with M22, confirm that perfusing brains with M22 for approximately 60 minutes at a temperature near 0 degrees C is sufficient to completely inhibit ice formation at lower

temperatures at typical cryonics cooling rates. In other words, a brain perfused with M22 under optimal conditions can be vitrified despite limited penetration of CPAs through the BBB. The cryoprotection that makes this possible is believed to be a result of the combined effect of penetrating CPAs that reach brain tissue and natural solutes in the brain (proteins and salts) whose concentration is increased by cell shrinkage as water leaves the brain in response to water activity gradients.

Warm or cold ischemia tend to eliminate brain shrinkage in response to cryoprotectant perfusion, presumably because ischemia compromises the BBB in a time-dependent manner. The compromised BBB allows CPAs to enter tissue faster so more of the water that leaves the brain can be replaced by CPA molecules, resulting in less shrinkage. One (ironic) consequence of this is that in cryonics severe dehydration (shrinkage) during cryoprotectant perfusion is often an indicator of good patient care (i.e. minimization or mitigation of ischemia).

In local Alcor cases that have been conducted under good conditions, severe dehydration was often observed through the burr holes. Recent Computer Tomography (CT) scans performed for Alcor have show the dehydration even more clearly. CPA-induced brain shrinkage can reduce brain volume by almost 50%. See Figure 18-1.

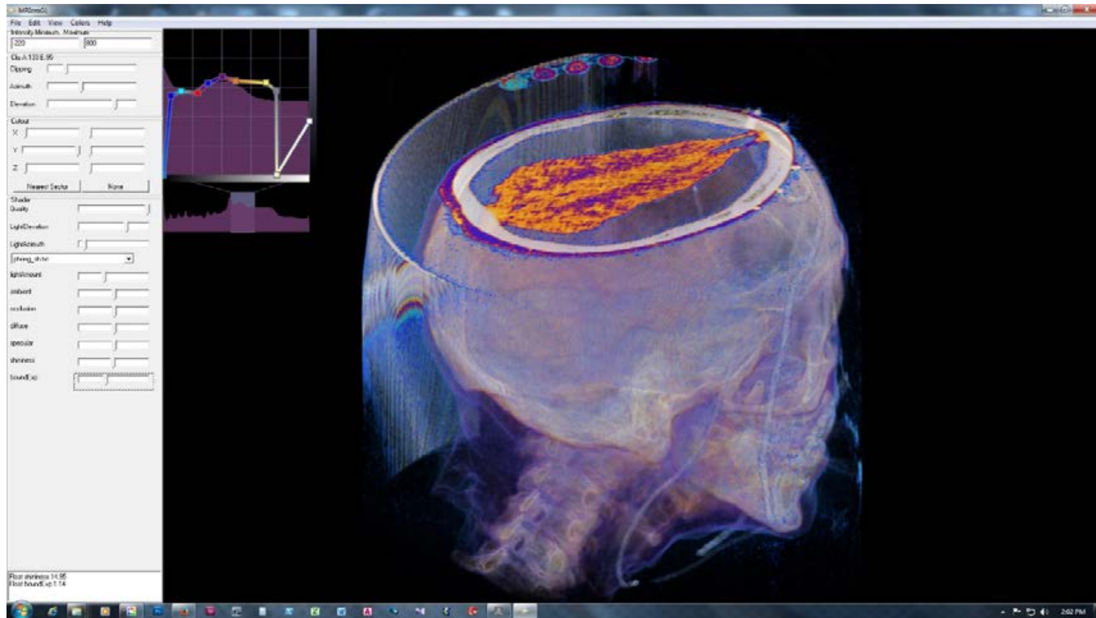

Figure 18-1. CT Scan of Alcor patient showing cryoprotectant-induced brain dehydration (orange area).

Cerebral dehydration (shrinkage) was identified as a potential form of injury in a case report for patient A-1097 (2006). At the Cryonics Institute, Yuri Pichugin also demonstrated that the extreme dehydration associated with modern vitrification solutions is not compatible with good brain slice viability. This is consistent with cryobiological theory, in which one of the ways that penetrating cryoprotectants are known to protect cells during cryopreservation is by preventing freezing-induced elevation of natural salts and proteins inside cells. (It is less toxic to cryoprotect cells by replacing water inside them with artificial CPAs like ethylene glycol than by removing water to increase concentration of natural salts by shrinkage.) Another reason that shrinkage is undesirable is that the appearance of shrunken brain tissue in electron micrographs is difficult to interpret and compare with control micrographs. In the case of M22, perfusion unloading has been shown to restore a more normal appearance of cells. However it would be desirable to minimize shrinkage during the entire cryopreservation process, especially if doing so mitigates toxicity as expected.

Osmotic opening of the BBB with molecules such as mannitol have a transient effect on BBB permeability but do not seem potent enough to permit brain cryoprotection without dehydration. The most promising approach at the moment is to use a BBB modifier to cryoprotect the brain without shrinking. Unpublished research has demonstrated the efficacy of adding a small concentration of detergents like SDS (sodium dodecyl sulfate) to the perfusate, a technique first pioneered by Yuri Pichugin at the Cryonics Institute. If there are no serious toxicity effects this would be a solution that Alcor could adopt in the future if a way can be found to adjust for effects of different amounts of cerebral ischemia prior to perfusion so that edema (brain swelling) would not result.

Cryoprotective Enclosure Design

Cryoprotection requires a stable and dedicated environment to conduct perfusion. The most basic incarnation of this idea is to use a surgical table to do both surgery and cryoprotection. The patient can be surrounded by ice packs during surgery and perfusion and after completion moved to a cooling box for cooling to liquid nitrogen temperatures.

Alcor uses a more sophisticated whole body patient enclosure that allows for surgical procedures, cryoprotection procedures, monitoring, and cooling. See Figure 18-2. This enclosure minimizes patient handling, cooling can be switched on or off in sectors for surgery visibility, cryoprotection and (initial) deep cooling are done in the same environment.

Figure 18-2. Whole Body Perfusion Enclosure.

This enclosure employs intermittent injections of nitrogen gas to maintain cold temperatures that are appropriate for different stages of cryoprotective perfusion, including temperature below 0 degrees C during later stages.

The enclosure has two independent cooling stages. First is a thin cold stage, constructed of a conductive metal. Cold vapor circulates from foot to head and returns in a counter-current manner within the cold stage, equalizing the temperature gradient along the length of the table. The vapor is contained in the cold stage, and not vented into the patient space, allowing cooling to

occur without any vapor to obscure vision. Second, vapor is circulated laterally through the patient compartment. This is divided into 4 independently-controllable zones, roughly corresponding to the legs and feet, groin, chest, and head, allowing independent access for femoral, thoracic, and head/neck surgery. Access is also provided to drill burr holes during perfusion and cooling. Temperature control is through Omega controllers, connected by Ethernet to the central computer system for monitoring and changing set points. If the central computer fails, the controllers will maintain the last set temperature.

The interior space of the cooling enclosure is constructed to match exactly the dimensions the whole body pods. This allows for optimal deep cooling in the same enclosure and will enable Alcor to immediately know if the size of the patient will create a problem for the typical storage pod.

The acrylic enclosure is secured on top of a stainless steel frame, constructed to fit on top of the surgical table. The frame prevents flexing of the acrylic top, and provides space for mounting equipment needed for the table. Data collection is mounted onto the frame, along with suction, electrocautery, and a gas manifold for pneumatic surgical and includes an uninterruptible power supply, allowing function to continue without power at least long enough to start our emergency generator.

The current whole body enclosure can drop to –110 degrees C and can be used for rapid cooling. Since there is no need to move the patient to a different cooling system, this enables commencement of deep cooling immediately after cryoprotective perfusion ends. The table can also be rolled to the cooldown bay during the latter portion of cooling if the OR is needed for another case.

Alcor has also developed a "neuro" enclosure with similar properties to be used during cryoprotective perfusion of neuropatients, which can be seen in Figure 18-3.

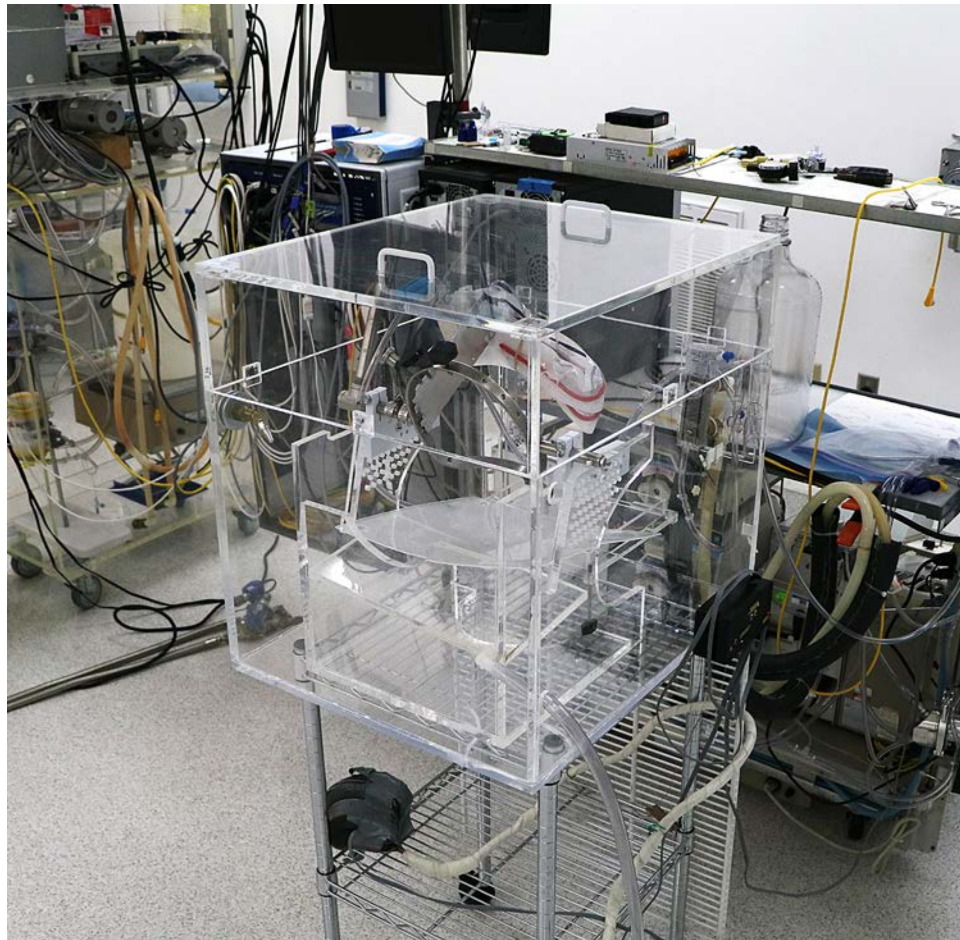

Figure 18-3. Neuro Perfusion Enclosure

Cryoprotective Circuit Design

The cryoprotective perfusion circuit should ideally be able to perform three objectives in this sequence: blood washout, cryoprotective perfusion, and cooling.

Washout

In cases where blood washout must be performed in Alcor's operating room (most local cases) it is desirable to separate this part of the procedures from the actual cryoprotective perfusion to minimize "contamination" of the circuit

with formed elements in the blood, inflammatory products, and other undesirable debris. To achieve this objective, the circuit needs to be designed to allow blood washout without using the cryoprotective circuit. This can be achieved by including separate inlet and discard lines for the washout that will leave the closed circuit cryoprotective perfusion part of the circuit “clean”. The procedure can be done as in conventional remote blood washout. In cases where the patient arrives at fairly high temperatures, however, recirculation with the washout solution might be desirable before exposure to the cryoprotective agent. For this reason, the washout part of the vitrification circuit should be able to be run closed circuit without contaminating the cryoprotective circuit.

Cryoprotective Perfusion

Basic cryobiology knowledge dictates that cryoprotective agents be gradually introduced to prevent large osmotic gradients that can damage cells. This is achieved at Alcor by starting cryoprotective perfusion with a “base perfusate” (aka carrier solution, aka vehicle solution) that contains no cryoprotectant ingredients. The perfusate enters the patient through the aorta or carotid arteries, and then venous effluent from the patient is carried to a recirculating reservoir, also called mixing reservoir. Perfusate is mechanically stirred in the mixing reservoir and then recirculated back to the patient. A cryoprotectant solution concentrate is added to the mixing reservoir at a controlled rate to slowly increase the concentration of cryoprotectants in the solution being pumped back to the patient. This creates what’s called a cryoprotectant “ramp.” The ramp isn’t a physical object. The ramp is the planned profile of increasing arterial cryoprotectant concentration as a function of time, which when graphed looks like a ramp because the initial stage is usually linear.

A diagram of the cryoprotectant circuit is shown in Figure 18-4.

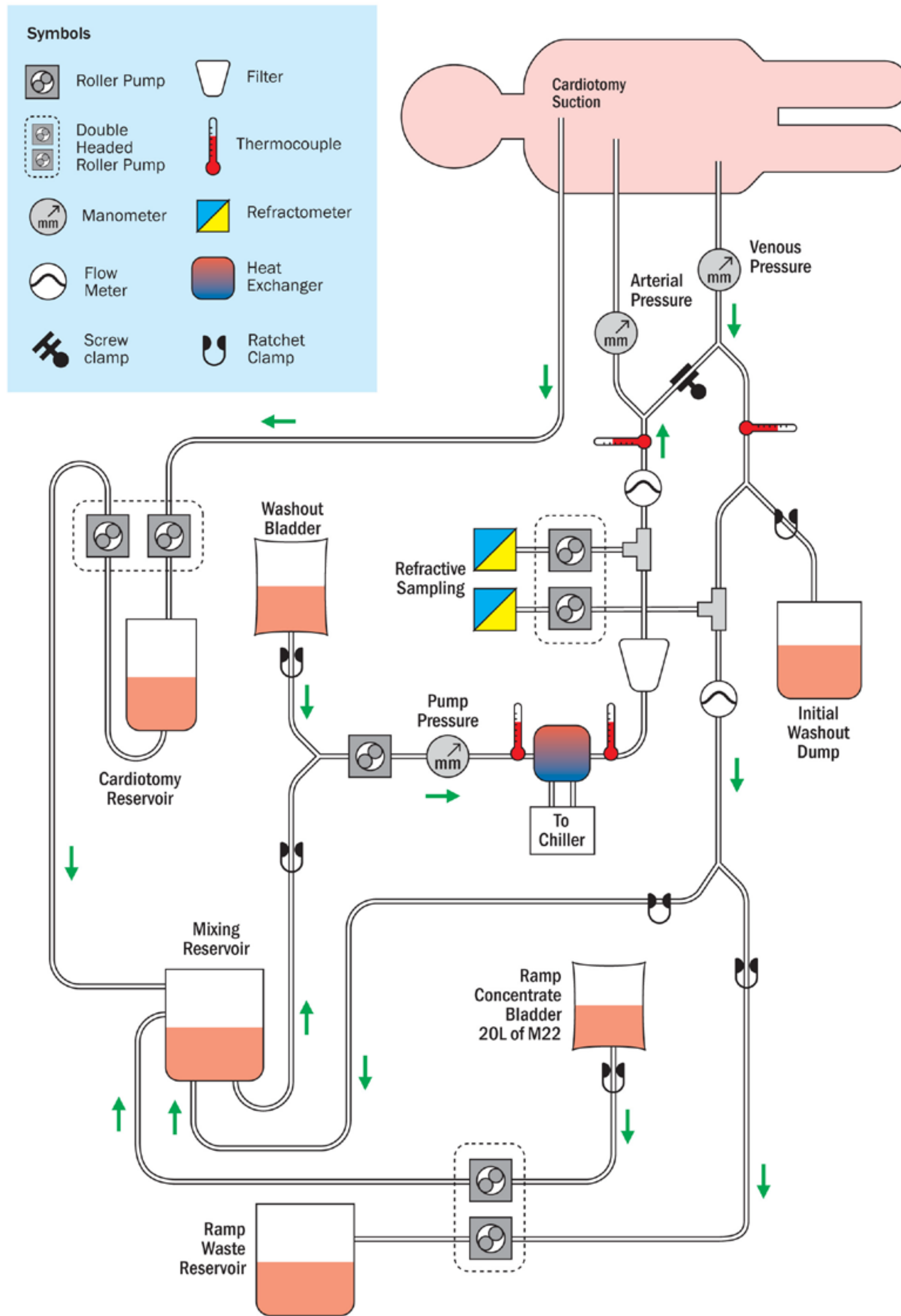

Figure 18-4. Recirculating cryoprotective perfusion circuit used at Alcor. The cannulation points are the thoracic aorta for the arterial line, and right atrium of the heart for the venous line.

As cryoprotectants replace water inside cells, the venous effluent leaving the patient and returning to the mixing reservoir has a slightly lower concentration of cryoprotectants than the solution going into the patient. This arterio-venous concentration deficit is further increased if the arterial concentration is increasing with time. However the arterio-venous concentration deficit will remain, even if the arterial concentration is held constant, until the concentration of cryoprotectant inside the patient equilibrates with the perfused arterial concentration. This concentration deficit is compensated by cryoprotectant concentrate solution being continuously added to the mixing reservoir so as to maintain the desired aterial concentration at every stage of the perfusion. Perfusate is discarded from the venous side of the circuit as necessary to maintain a constant reservoir level as concentrate solution is added.

The perfusion circuit design described above is called a "closed circuit" system. Closed circuit perfusion is the state-of-the-art perfusion method used in cardiopulmonary bypass for heart surgeries (without cryprotectant), organ cryopreservation research, and that is also preferred for cryonics. Its principal advantage is that the cryoprotectant concentration can be controlled continuously over as long a time as is necessary to achieve effective cryoprotection of tissues without excessive consumption of perfusate. It also minimizes use of expensive cryprotectant solutions and greatly reduces perfusate waste.

An alternative to a closed circuit with a mixing reservoir is to introduce the cryoprotective agents in a series of increased concentrations, without recirculating the perfusate. This is commonly known as an "open circuit" system. This simpler method is currently used by Alcor for a procedure called field cryoprotection. How such a method compares to closed circuit perfusion depends on the number of steps (perfusates with different concentration) at which the cryoprotective agent is introduced. An advantage of introducing the agent in such a manner is that residual blood cells and products of cell lysis will not be recirculated. Another advantage is that operation of the perfusion circuit will be easier because a number of components, such as the mixing reservoir, can be eliminated from the circuit. A major disadvantage is that

such open circuit perfusion will require much larger amounts of the perfusate, which is not desirable in light of the costs of agents like B2C and M22. Another disadvantage is the frequent changing of perfusate bags that such a system requires, increasing the probability of errors such as introducing air emboli into the system. It is most practical for neuropatients (or cryoprotection of only the head of whole body patients) at locations too far away to transport at hypothermic temperatures to Alcor to benefit from a more controlled perfusion in Alcor's operating room.

Minimizing Perfusate Loss

In whole body cryopreservation another component of the vitrification circuit is the "cardiotomy sucker" and reservoir that is used to return perfusate to the circuit that is leaks into the thoracic cavity during perfusion as a result of surgical access to cannulate the heart and surgical wounds. It's generally preferable to discard suctioned perfusate, but it may be returned to the circuit if the loss is large.

Refractive Index Measurements

In a closed circuit system, the concentration of cryoprotectant is measured by instruments that measure the refractive index of perfusate. The refractive index may then converted to cryoprotectant concentration by a calibration scale that is specific to the cryoprotectant solution being used. Alcor practices the convention of recording refractive index readings in Brix (a scale widely used in the food industry), and then managing the perfusion based on reaching a target Brix value that corresponds to the desired target cryoprotectant concentration.

Inline refractometry requires modification of the circuit, depending how the refractive index of the perfusate is monitored during cryoprotective perfusion. The current state of the art is to complement taking intermittent refractive readings from the arterial and venous side with inline refractometry to monitor trends over time. Important decisions (such as ending cryoprotective perfusion) are usually made by consulting the readings of a handheld or benchtop refractometer.

Inline refractometry affects cryoprotectant perfusion circuit design because a choice needs to be made of whether to equip the circuit with the ability to control the temperature of the perfusate to produce reliable refractometry readings. Because the temperature of the perfusate varies as cryoprotection perfusion progresses, and the refractive index of solutions varies with temperature, and the magnitude of this variation increases with concentration, it is desirable to obtain temperature adjustment of inline refractometry readings. Basic inline refractometers have internal temperature compensation circuits that provide temperature correction that is only exactly correct at one particular concentration. The most expensive industry inline refractometers have user-programmable temperature compensation coefficient tables that can be configured for accurate temperature compensation at all concentrations across a temperature range. An alternative to temperature compensation is to install inline refractometers into bypass lines in which small quantities of perfusate are sampled and temperature conditioned before passage through the refractometers. However this makes the perfusion circuit substantially more complicated. Imperfect temperature compensation is the primary reason why offline precision refractometer measurement of perfusate samples is the gold standard for determining if concentration targets have been reached.

The circuit contains a separate washout “circuit” and hookup to reduce contamination of the circuit with blood.

The circuit includes separate, optional arterial and venous lines that direct a small portion of the perfusate to a warming bath prior to entering the inline refractometers. The perfusate can be discarded in the waste reservoir.

The last optional part of the circuit is the cardiotomy sucker and reservoir. Returning cardiotomy suction to the circuit is undesirable, but may be necessary to prevent depletion of circuit volume if suction drainage is faster than the rate of cryoprotectant concentrate addition.

Oxygenator

Though most extracorporeal heat exchangers come with an oxygenator, oxygenation is presently only considered of value during higher temperature

perfusion, such as during blood washout and cooling to toward 0 degrees C. Although future research may show otherwise, oxygenation is presently considered not to be indicated during cryoprotectant perfusion.

Stir Plate and Stir Bar

The concentrate is added to the base perfusate in the mixing reservoir and needs to be mixed before delivering it to the patient at the arterial side. The most important considerations for the stir plate and mixing reservoir are: the set-up should be physically stable (the stir bar should stay in the middle and not spin out of place); mixing needs to be vigorous, instantaneous, and homogeneous; and dead space needs to be reduced.

In case an "unconventional" choice for a mixing reservoir is made (for example, glass or a plastic that has not been used before) it is important to validate the stir plate / mixing reservoir setup by running experiments with a suitable aqueous solution for issues like mixing efficiency, homogeneity, stability of the stir bar during prolonged use and related issues.

Eliminating Foam and Air Bubble Formation

Because the concentrate has a higher specific gravity than the base perfusate, vigorous mixing is required to prevent the concentrate from sinking to the bottom of the mixing reservoir. But the requirement of vigorous and instantaneous mixing introduces different risks such as foam and air bubble formation. Air bubbles can be introduced to the perfusate through the stirring-induced air vortex. As the viscosity of the solution increases, saturated air bubbles can induce foam formation. This phenomenon is further aggravated by some of the polymers in the latest vitrification solutions (as evidenced by serious foam formation during the final stages of the cryoprotection of patient A-1049 in 2006). To some degree these problems can be reduced by maintaining a high level of perfusate in the mixing reservoir and limiting the RPM (rotations per minute) to the minimum required to produce adequate and homogenous mixing of the perfusate. A specific solution to (micro) bubble formation is to equip the reservoir with a "floating lid" to prevent generation

of an air vortex. This lid can be made of similar (or related) material and should allow the addition of weight or a (sterile) fluid to prevent wobbling and tipping of the lid. Foam formation can be eliminated by coating the bottom of the lid with defoamers / antifoamers.

Another solution to the problem is to include a reservoir between the mixing reservoir and the arterial pump that has an antifoaming coating. Although such a coating has been implemented with success in the past in cryonics, adding another reservoir (if a reservoir is added to the venous side as well) may complicate the circuit even further and might produce more risks than benefits. The combination of prudent stirring speed, a floating lid and an effective arterial filter should be able to reduce most of the concerns. Some basic experiments to determine the degree to which the circuit needs to be modified to eliminate (micro)bubbles and foam.

Heat Exchanger

A heat exchanger is part of the vitrification circuit to do washout and cryoprotective perfusion at hypothermic temperatures and to cool the perfusate to high subzero temperatures during the final stages of cryoprotectant perfusion. The ideal heat exchanger for a vitrification circuit has excellent heat conductivity, is chemically resistant, can tolerate thermal stress during operation, and is made to operate under subzero temperatures. Although heat exchangers usually come combined with an oxygenator, there are separate medical and industrial heat exchangers on the market that are more suitable

Arterial filter

A 40 micron filter is included in the vitrification circuit to remove air bubbles and particulate matter. To be on the safe side a bypass line can be added to the filter to deal with a dysfunctional (clogged) filter. The filter can be bypassed by simply clamping the line inferior to the filter. Cryoprotective perfusion can be continued without the filter or a new filter can be cut into the line (when it does not have flow) and perfusion through this filter can be continued.

A 0.2 pre-bypass filter can be added to the circuit to filter the cryoprotectant concentrate prior to entering the mixing reservoir. One advantage of this setup is that particulate matter (such as sedimentation or plastic particles) that have accumulated during storage of the perfusate can be removed prior to perfusing the patient. Because the diameter of red blood cells is larger than the pores of a 0.2 micron filter, such a filter should never be placed in the recirculating part of the vitrification circuit, but between the cryoprotectant addition pump and the mixing reservoir.

There are at least two problems with adding a pre-bypass filter to the circuit: 1) This will introduce differences in flow rate between the addition and discard line of the circuit if the same pump is used); and 2) The pre-bypass filter can get clogged as a result of a high particulate matter and/or the perfusate itself. A pre-bypass filter in the refractometry line, to remove particles before measurements, could also be beneficial but in light of residual blood in the venous line a 40 micron filter is advisable in this case as well.

Sample Ports

The arterial and venous lines should be equipped with sample ports to take blood and perfusate samples during washout and cryoprotective perfusion. Although professional perfusionists prefer not to sample directly from the arterial and venous lines, the “reservoir and gang of stopcocks-method” they utilize is too complicated for the average vitrification circuit operator and may actually be riskier as a result. There should be at least three sample ports in the circuit: 1) arterial line, 2) venous line proximal to the patient for sampling during washout, and 3) venous line distal to the patient for sampling of the perfusate during recirculation. One other possibility is a sample port between the cryoprotectant addition pump and the mixing reservoir to sample the solution for quality control measures. A sample port in the reservoir is also a good idea to assess the quality of mixing. The sample ports can be created inserting a connector with a stopcock and luer-lock through which samples can be taken by screwing in a syringe that can be filled and closed by turning the stopcock.

Temperature

Temperature is measured at the arterial and venous site and can be measured inline using a thermocouple which can be either hooked up to a temperature logger or software. Temperature can be further measured in the patient at locations such as in the nose (nasopharyngeal) and the brain. If a warming bath will be added to the vitrification circuit, temperature measurements need to be taken there as well.

Refractometry

Refractometry, as far as it concerns circuit design considerations, has been discussed in some detail above. Refractive index measures should be taken continuously inline and intermittently from the sample port.

There are a lot of handheld and benchtop refractometers on the market. In the past cryonics organizations have used inexpensive handheld refractometers. Both major cryonics organizations have now abandoned such refractometers in favor of more expensive (used) benchtop and handheld refractometers. These refractometers are robust, reliable and more suited for cryonics purposes, and some of them feature integrated data collection and laptop connections.

Pressure

A manometer and transducer can be used to measure pressure in the circuit to allow for manual adjustments of flow rate. The current system at Alcor uses automated pressure data collection which is used by the software to control pump speed.

One limitation of measuring pressure only in the circuit is that it does not take into account the pressure changes (drops) that are produced by the cannulae. One solution to this problem is use of an arterial perfusion cannula with integral pressure monitoring line.

Pumps

Any kind of (medical) roller pump that can generate sufficient flow for cryoprotective perfusion can be used. If the perfusion circuit is equipped with software to monitor and control perfusion, pumps that allow speed control by external signal are necessary.

One issue that should be given some thought during development of a perfusion circuit is whether pulsatile flow is desired. There is now a wealth of literature on the potential benefits of generating pulsatile flow during cardiopulmonary bypass and a review of such literature might be warranted. The benefit of using pulsatile flow cryoprotective perfusion remains a topic of discussion. Older case reports of Alcor cases document the rational and use of this technology.

Waste Reservoirs

Unlike the mixing reservoir, the choice for waste reservoirs does not require much research. Any type of water jug or plastic reservoir should suffice. It is recommended, however, to have a separate washout and cryoprotective perfusion waste reservoir in case there is a scenario where perfusate from the waste reservoir needs to be re-introduced to the circuit. In such an (unfortunate) scenario it is desirable that the perfusate is not mixed with the blood and toxic elements that were initially washed out.

Cryoprotective Perfusion Cooling Options

During washout and the first stages of cryoprotective perfusion the perfusate can be cooled by running it through a heat exchanger that is supplied by ice water. Cooling can be enhanced by keeping the perfusate cold prior and during cryoprotective perfusion. Cooling can be further enhanced by placing the vitrification set-up (or most of it) in a refrigerated area. When the temperature needs to be dropped to subzero temperatures there are basically three options: 1) Adding salt or antifreeze to an ice water bath that supplies coolant to a heat exchanger. 2) Using an industrial chiller that uses mechanical

refrigeration to cool an antifreeze coolant solution (such as ethylene glycol solution) as a circulating fluid for the heat exchanger. 3) A chiller that uses liquid nitrogen (LN2) to cool the coolant. Experiments started at Suspended Animation by Mathew Sullivan and later adapted by Alcor culminated in the development of a custom-built LN2-driven chiller which is still in use today to cool the perfusate lines and the patient enclosure. See Figure 18-5.

Figure 18-5. Alcor Liquid Nitrogen Chiller

Whole Body Cryoprotective Perfusion Circuit Operation

Preparation for Perfusion

Each new whole body case requires a new sterile tubing pack. The only components in the circuit that are re-used are the pumps, the mixing reservoir, the refractometers, and the electronics to collect data during cryoprotective perfusion. Alcor should have at least 2 sterilized tubing packs available for

whole body cases at any time. After a case, OR supplies should be re-stocked and a new whole body circuit should be installed, strung, and checked (but not run) to restore cryoprotective perfusion capabilities as soon as possible. An experienced person should allow for at least 4 hours (including ½ to 1 hour for connecting the electronics) to install, string and check a new perfusion circuit.

The first step in preparing the circuit for cryoprotectant perfusion is to prime the circuit with the washout solution and base perfusate: B1. The washout solution comes in 20 liter bags and one bag is generally sufficient to wash out the blood of the patient. If the patient's blood has already been washed out during remote stabilization procedures, a modest initial washout is still recommended. This will also ensure that no expensive cryoprotective perfusate is wasted in a futile cryoprotective perfusion attempt if it is determined that perfusion of the patient is not feasible. The B1 washout perfusate should be sterile but should be checked for mold growth because this solution is a fertile growth medium for micro-organisms when sterility has been breached.

Before the circuit is primed with B1 all the connections should be checked to ensure that are no leaks in the circuit or components that have not been properly secured. Priming of the circuit is initiated by filing the Mixing Reservoir with B1 up to 10 liters, circulating it through the complete circuit, excluding the Cardiotomy Reservoir line and the Waste Reservoir lines. During priming the lines to the Waste Reservoirs and the Cardiotomy Reservoir line are clamped. During priming air bubbles are eliminated from the circuit, the various components are checked and the target temperature of the perfusate is lowered to approximate 0 degrees Celsius (but not lower!).

Blood washout

When the system is primed and maintained at the right temperature, washout of the patient is initiated when the circuit is connected to the patient's cannulae and the surgeon indicates that the line to the patient can be unclamped. During washout only a portion of the complete circuit is used, and the venous effluent is dumped in a separate waste reservoir. If the initial blood washout is used to take a venous sample of the patient, it is important to take this sample as soon as possible to ensure that the blood sample will not be

diluted with the washout solution. Initial washout of the patient should be continued until the venous effluent of the patient shows a consistent clear color or the perfusate has been exhausted. If no acceptable washout is possible due a severely compromised vascular bed and/or edema, the team leader and perfusionst can decide to decide to terminate any attempt at cryoprotective perfusion of the patient.

Cryoprotective Perfusion

Cryoprotective perfusion of the patient requires two different solutions. A carrier solution (base perfusate) that will be the starting perfusate in the Mixing Reservoir, and the M22 concentrate for whole body patient in a carrier solution that will be gradually added to the Mixing Reservoir. It is important to remember that the M22 concentrate also includes the carrier solution to avoid diluting the carrier solution ingredients in the mixing reservoir when more concentrate is added.

The M22 concentrate for whole body preservation and M22 concentrate for neuro preservation are not the same. M22 concentrate for whole body patients has an additional component to mitigate edema during perfusion. It is therefore mandatory to verify if the right solution has been chosen for the patient. The concentrate comes in a higher concentration than is necessary to vitrify (such as 1.2 or 1.25 times the published nominal full concentration M22) to ensure that the target concentration in the patient will be achieved within a reasonable period of time.

To initiate cryoprotectant perfusion the mixing reservoir should contain at least 15-20 liters of B1 (the carrier solution). During perfusion the M22 concentrate is gradually added to the reservoir and rapidly mixed through the use of a large magnetic stirring bar. The proper position and functioning of the stirring bar should be monitored throughout perfusion to ensure that proper mixing of the carrier solution with the concentrate is taking place. During perfusion the level in the mixing reservoir should be monitored to avoid the reservoir running dry and introducing air into the patient. The perfusate level in the Mixing Reservoir should not be allowed to drop below 7 liters.

Watching and documenting the level of the mixing reservoir is one of the tasks of a scribe in the OR. If the level of the mixing reservoir starts dropping

to unacceptable levels, the perfusionist can increase the pump speed of the pump that supplies the concentrate to the reservoir and clamp off the discard line to return more perfusate to the mixing reservoir. If the mixing reservoir is observed to be in real danger of running dry the pumps should be stopped and perfusion halted until the perfusionist has restored enough volume to the mixing reservoir to safely resume cryoprotective perfusion.

M22 concentrate is gradually introduced to the mixing reservoir by a dual head pump that also controls the discard line. After mixing in the mixing reservoir the perfusate runs through the heat exchanger and filter to the arterial side of the patient. After the perfusate is exchanged for water in the patient the effluent returns on the venous side where most of the perfusate is returned to the mixing reservoir and a portion is discarded.

During cryoprotective perfusion the following parameters are being monitored: system pressure, arterial perfusion line pressure, arterial temperature and the refractive index. The refractive index is being monitored in-line and displayed on the computer screen and manual refractometry samples are taken. As a general rule, the inline refractometers are used to monitor trends and the manual samples are used to make important decisions such as decreasing the temperature for the second half of perfusion and terminating cryoprotective perfusion.

Subzero Cryoprotective Perfusion

When 50% of target arterial concentration is reached after 90 minutes, as evidenced by manual refractometry readings, the ramp is paused to hold the arterial concentration steady. Concentrate is only added as necessary to hold the arterial concentration at a plateau of 50% of target while the venous concentration catches up. During this time, the chiller coolant temperature is dropped as to necessary to decrease the perfusate temperature in the arterial line from +3 degrees Celsius to -3 degrees Celsius. After holding the 50% plateau for a minimum of 15 minutes, and only after an arterial temperature of -3 degrees Celsius is attained, concentrate addition resumes to raise the arterial concentration to 105% of target concentration as quickly as possible without overshooting. The target concentration is also called CNV or “concentration needed to vitrify” (currently the published nominal concentration of M22

vitrification solution). When the arterial concentration of 105% CNVhas been achieved as measured by manual refractometry, concentrate continues to be pumped at whatever rate is necessary to maintain this arterial concentration plateau until the venous concentration rises to 100% CNV. The plateau should be maintained for a minimum of 60 minutes and a maximum ideally not exceeding 90 minutes.

In some cases, Alcor will be able to initiate cryoprotective perfusion but will progressively encounter poor perfusion and increasing edema. If these problems cannot be overcome through surgical adjustments, Alcor can decide to terminate perfusion. Cryoprotective perfusion is not an all-or-nothing operation and in some circumstances Alcor may only be able to reach lower target concentrations.

Pressure

Recommended arterial perfusion pressure for whole body patients is between 80 mmHg and 100 mmHg and should not exceed 120 mmHg. This is especially important in the case of patients with ischemic exposure or prolonged cold transport times. Perfusion pressures should be carefully monitored. If the perfusion pressure is not controlled by software it is important to be aware of the fact that pressures will respond to temperature and CPA concentration and tend to rise quickly during the final stages of perfusion when the temperature is dropped below zero and the viscosity of the solution increases. Ideally arterial line pressures should be corrected for pressure loss in cannula, if known, to achieve the desired target pressures intra-arterially.

After cryoprotective perfusion is completed, the patient may be removed from the enclosure for deep cooling or may remain in the enclosure for initial stages of deep cooling, depending of the design of the enclosure.

Negative Pressure for Venous Return

As is customary with clinical cardiopulmonary bypass, Alcor does not use a pump to return venous blood from the patient to the mixing reservoir. This means that venous pressure will rise inside a patient’s veins to whatever pressure is necessary to overcome flow resistance through the cannula and

tubing between the right heart and mixing reservoir. During normal blood circulation, veins have a very low pressure inside them. If pressure inside a patient's right heart and vena cava is allowed to rise to more than just a few mmHg, systemic edema and filling of the lungs with perfusate is likely to result. The most effective blood washout and cryoprotective perfusion, with least edema or pulmonary leakage, will result from maintaining venous pressure as close to 0 (zero) mmHg as possible.

Venous pressure at the venous cannulation point can be minimized by placing the venous return reservoir (mixing reservoir) near the floor, several feet below the patient. This creates a negative pressure, or suction, to offset the positive pressure that would otherwise be necessary to move perfusate through the venous cannula and venous return lines. Ideally the reservoir should be on a table of adjustable height (e.g. "Lab Jack"). The reservoir height should be adjusted until the venous cannula is observed to "chatter" (alternately suck closed and then open), and then slightly raised. This ensures that the venous pressure inside the right atrium is being maintained at near zero pressure, which is nominal. The reservoir level necessary to minimize venous pressure should be checked and adjusted throughout the perfusion. If "chattering" cannot be achieved even with a maximum height difference between the patient and reservoir, larger venous cannula, wider tubing, or otherwise reduced flow constriction in the venous lines might be indicated.

Note that for suction to be effective, the venous lines between the patient and reservoir must be primed full of fluid. Air should not be allowed to enter and "deprime" the venous lines. If air enters the venous return lines, the lines must be reprimed immediately to avoid buildup of dangerous venous pressure inside the patient. Priming is easier if sufficient slack exists in the venous lines for an "S" to be made by the perfusionist to "walk" air through the lines to the reservoir.

Neuro Cryoprotective Circuit Operation

Whether there is a distinction between a whole body perfusion circuit and neuropatient perfusion circuit depends on surgical protocol and patient enclosure. In neuropreservation protocols in which the cepehalon (head) is

removed only after cryoprotection of the upper part of the body, the environment and conduct of perfusion is identical to whole body cryoprotection with the exception of clamping the descending aorta (and extremities) to direct as much of the perfusate as possible to the head. In such protocols, when the target concentration of the cryoprotectant has been reached, the cephalon is surgically removed (without warming the tissue) and placed in a cooling box for rapid deep cooling. This method was used by Alcor for cryoprotection of neuropatients during the 20th century.

Current Alcor protocol for neuropreservation is to separate the head from the rest of the body prior to starting cryoprotective perfusion. The cephalon is secured in a cephalic enclosure and the carotid vessels are cannulated. Venous perfusate can be returned from the jugular veins, or can be drained into a tray and returned to the recirculating reservoir, which is the current procedure. A major advantage of the isolated cephalon perfusion method compared to older techniques described above (or contemporary whole body perfusion) is that allowing severed jugular veins to freely drain reduces venous pressure inside the brain, thereby increasing perfusion flow rate and decreasing the time necessary at the peak arterial cryoprotectant concentration plateau for the target concentration in the venous effluent to be reached. Lower venous pressures are also expected to reduce the severity of cerebral edema that sometimes occurs in patients who suffered long periods of cerebral ischemia before the start of cryoprotectant perfusion.

The circuit that is used for perfusion of the cryoprotectant solution basically reflects the same design as whole body circuits. A roller pump withdraws the CPA concentrate from a reservoir and feeds this into the recirculating reservoir where the CPA is mixed with the carrier solution. A second pump forces the perfusate through a 40 micron filter and heat exchanger into the patient.

Cooling of the perfusate below zero degrees Celsius can be achieved by using a heat exchanger supplied by coolant fluid from a sub-zero chiller. Alcor’s current setup uses an LN2-driven chiller for both perfusate and neuro enclosure temperature control.

Computer Control and Data Collection

There are three basic possibilities for control and data collection during cryoprotective perfusion:

- Manual data collection and manual cryoprotection control
- Automated data collection and manual cryoprotection control
- Automated data collection and automated cryoprotection control

In theory, it would be possible to have automated perfusion control and manual data collection but, as a rule, a general technology to control perfusion requires automated data collection to do its job.

Since its inception Alcor has moved from mostly manual cryoprotection control and manual data collection to manual control of perfusion and automated data collection. More recently, Alcor has developed a system that also conducts perfusion.

The following is a list of potential characteristics of an automated perfusion and cooling system.

- Control of flow rate according to arterial pressure.
- Monitor and display temperatures, and control the chiller coolant temperature based on arterial temperature.
- Monitor and display cryoprotectant concentration (refractive index).
- Control ramp by separate volumetric addition of CPA concentrate and base perfusate. Separate addition of concentrate and base will avoid situations where making up table losses results in too-rapid increases in CPA concentration.
- Control mixing reservoir level.
- Monitor volume and concentration of ramp waste reservoir.
- Bubble and level alarms

- Dissolved oxygen and ion selective electrodes.
- Suspended solids meter (for quantifying emboli)

The more complex and "autonomous" such a software-controlled system is, the more important it is to include a failure mode to (temporarily) stop software control revert to manual control of pumps and pressure monitoring.

Surgery

Surgical procedures associated with cryoprotection procedures include:

- Surgery to obtain access to the patient's vascular system to wash out the blood.
- Surgery to obtain access to the vascular system to perfuse the patient with a cryoprotectant.
- Surgery to create small "burr holes" in the cranium to visually validate cryoprotectant perfusion and observe changes in brain volume.
- Surgery to isolate the cephalon from the body

Surgery Preceding Whole-Body Cryoprotection

In whole-body cases, the surgeon performs a median sternotomy and places an arterial cannula in the aorta and a venous cannula in the right atrium for venous return.

Alternatively, it's theoretically possible to conduct a whole body cryoprotective perfusion using femoral cannulae that are sometimes used for field blood washout of remote cases prior to transport to Alcor. However femoral cryoprotectant perfusion is difficult because femoral vessel and cannulae diameters are too small to deliver the necessary flow rates without very high back pressures. Systemic venous pressures would also be undesirably high. (Limited open-circuit femoral whole body cryoprotection was done once in the field for Alcor patient A-2158, with venous drainage facilitated by the cephalon being already severed for faster transport to Alcor

ahead of the rest of the body due delays obtaining an interstate transit permit for human remains.)

Surgery Preceding Neuro Cryoprotection

Although it's theoretically possible to use the same surgical technique as for whole body patients, this would not not only be very time inefficient but also substantially increase the cost of the cryoprotectant. For this reason, in surgery for neuropreservation patients cannulation techniques are used to ensure that only the head and brain will be perfused. Several different approaches are known in cryonics.

Option 1 (used only historically by Alcor). The surgeon performs a median sternotomy and places an arterial cannula in the aortic arch and a venous cannula in the superior vena for venous return. Systemic and upper body perfusion can be prevented by clamping the descending aorta and placing tourniquets on the arms. A more sophisticated approach to using tourniquets would be to ligate the subclavian arteries distal to the vertebral arteries. Venous return from the extremities can be prevented by ligating the left and right innominate veins distal to the left and right internal jugular veins. See Figure 18-6.

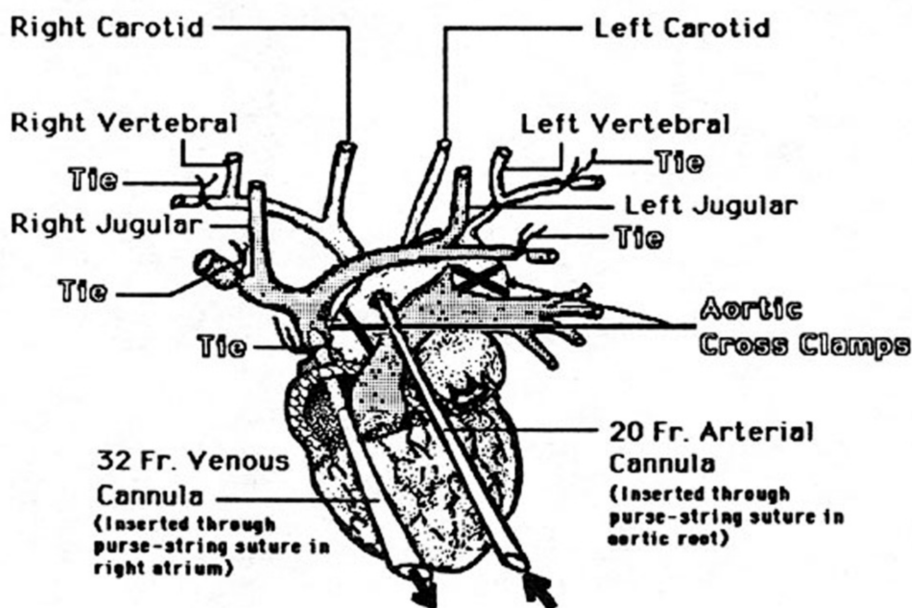

Figure 18-6. Cannulation of vessels near the heart.

After completion of perfusion, a circumferential skin incision is made at the base of the neck extending anteriorly and posteriorly to just below the margins of the clavicle. The skin is dissected from the underlying connective tissue up the level of the 5th cervical vertebra to form skin flaps. The muscles of the neck are cut with a #10 scalpel down to the junction of the 5th and 6th cervical vertebrae. The cephalon is removed from the body using a Satterlee or Gigli saw by cutting between the 5th and 6th cervical vertebrae.

Option 2. An alternative approach that has been practiced by the Cryonics Institute is to perfuse all the vessels supplying blood to the brain by cannulating just proximal to the bifurcation that perfuses both the vertebral and carotid arteries, ligating the subclavian artery distal to the vertebral on the right side of the body, and individually cannulating the vertebral and carotid arteries on the left side of the body. See Figure 18-7.

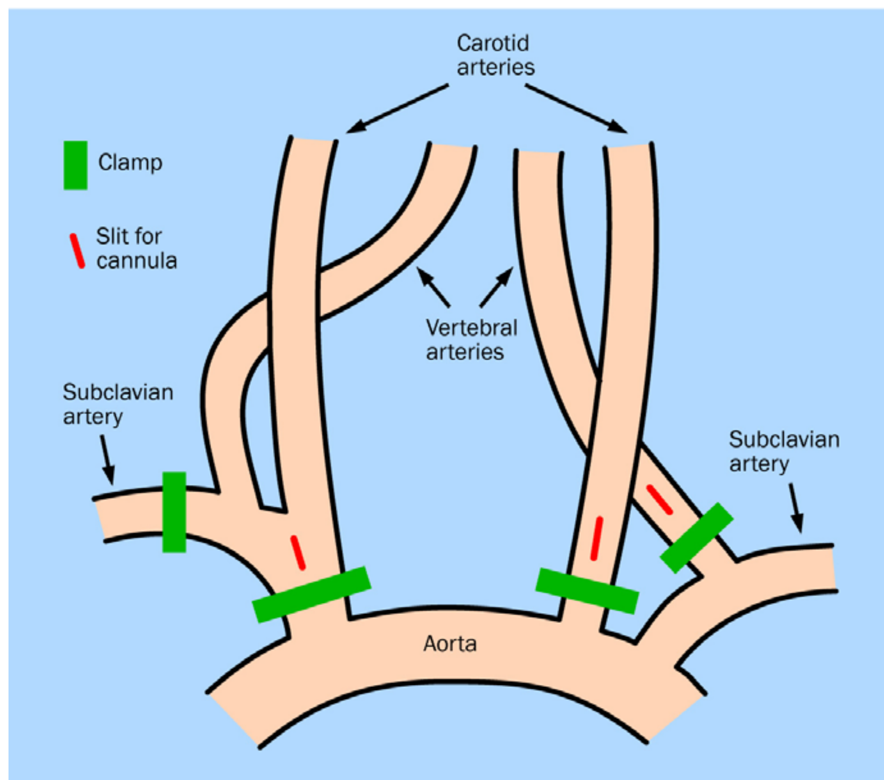

Figure 7. Surgical technique used at the Cryonics Institute.

Option 3. The alternative that is currently practiced at Alcor is to cannulate the carotid arteries, remove the cephalon (cephalic isolation), and perfuse the head through the carotid cannulae while the head is held in a specially designed temperature-controlled enclosure. One drawback of this technique is that in some patients the internal carotids (that supply blood to the brain) branch from external carotids (supplying blood to the scalp and face) below the level of the clavicle in the chest, which can reduce or eliminate perfusion of facial tissues. Whether both the carotid and the vertebral arteries need to be cannulated depends on whether the patient has an intact Circle of Willis. If there is reason to suspect that the patient does not have an intact Circle of Willis (i.e. no effluent observed from vertebrals during carotid perfusion), cannulating all four major cerebral vessels is required. Otherwise the vertebral arteries are clamped after observing effluent from them so that arterial pressure inside the Circle of Willis isn't reduced by open vertebrals.

Burr Holes

Burr holes are drilled in the skull by a neurosurgical tool called a perforator to monitor when the brain shrinks (normal response) or swells in response to cryoprotectant perfusion. After shaving the head two 3-5 cm scalp incisions are made 2 cm from the midline on each parietal lobe using a scalpel blade. The scalp is retracted and a bone scraper is used to remove tissue from the scalp. Two burr holes are made using a surgical device known as a perforator, while squirting sterile saline to cool the perforator and tissue. A rongeur can be used to to enlarge the holes, if necessary. The dura mater is opened using a dura hook (to retract the dura away from the brain) and iris scissors or a scalpel blade. Thermocouples and a "crackphone" (acoustic microphone to monitor fracturing during cryogenic cooldown) can be placed between the skull and the dura. After cryoprotectant perfusion, the burr holes are filled with bone wax and the incisions are closed with staples.

Monitoring Cryoprotective Perfusion

Whereas patient monitoring in stabilization procedures (like blood sampling) is mostly used to improve and guide future cases, during cryoprotective perfusion monitoring generates data to actually conduct procedures. For example, arterial pressure readings are used to change pump speed to maintain a pressure target. Temperature readings are used to determine the start of cryoprotection. Refractive index measurements determine when it is time to accelerate the ramp and terminate perfusion. If the patient suffered significant ischemia prior to perfusion then monitoring weight gain, local edema, and the brain through the burr holes provide information on whether to continue or stop cryoprotection. Without monitoring for cerebral edema to determine if perfusion must be stopped, a brain could herniate through the foramen magnum during cryoprotectant perfusion.

Alcor protocol dictates that in case of isolated head perfusion the cephalon should be weighed prior and after completion of cryoprotective perfusion to determine the degree of dehydration or edema (and even infer the degree of ischemia). For whole-body patients the patient enclosure can be modified to include scales to weigh the patient prior and after cryoprotection as well (for example, during cryoprotective perfusion of Alcor patient A-1108 a bed scale was used to collect data on weight changes and the amount of weight gain was reported in the write-up). Considering the tendency of whole body patients to gain weight during cryoproectant perfusion in cases of (extensive) ischemic exposure, documenting weight gain is important for good case reporting and meta-analysis.

Samples of the venous effluent can provide more information than just the refractive index. Manual and in-line measurements of electrolytes, metabolites, proteins, and dissolved oxygen can be done on perfusate during various stages of cryoprotection. As a general rule, not much is currently known about the chemical composition of the venous effluent of patients undergoing cryoprotective perfusion.

Tissue samples can be taken and subjected to real-time viability assays (LDH, K/Na etc) and/or prepared for electron microscopy. In this case of the

brain, microsamples of brain can be taken upon completion of cryoprotective perfusion to determine the metabolic and fine structure of the brain.

In 2011, Alcor started doing x-ray CT scans on neuropatients after completion of cooling to liquid nitrogen temperature. During the transport to and from the imaging center, and during the imaging process, patients remain safely immersed in liquid nitrogen in their containers. Originally intended to verify “crackphone” placement, the technology has been found to hold promise to determine regional cryoprotectant uptake and ice formation. The scans can also be used to look at the degree of brain dehydration (or lack thereof). The cephalon remains in its aluminum container under liquid nitrogen during the scan. In 2018 Alcor took delivery of its own in-house CT scanner.

This technology takes advantages of the differences between frozen, normal, and cryoprotected tissue. First, ice formation increases space between atoms because ice is less dense than liquid water. Unfrozen tissue therefore has a higher CT density than frozen tissue. Second, solutions of cryoprotectant chemicals have a higher physical density (more electrons per unit volume to scatter x-rays) than water. CT density therefore increases as cryoprotectant concentration increases. Finally, the cryoprotectant solutions used by Alcor contain dimethyl sulfoxide (DMSO), which contains the element sulfur. The higher atomic number of sulfur compared to oxygen in water makes it better at photoelectric absorption of x-rays.

The CT images allow Alcor to infer the quality of a cryopreservation on the brain and build a set of images that can be discussed in the context of other variables such as normothermic and cold ischemia, duration of patient transport, conduct of cryoprotective perfusion, etc. A sample scan is shown in Figure 18-8.

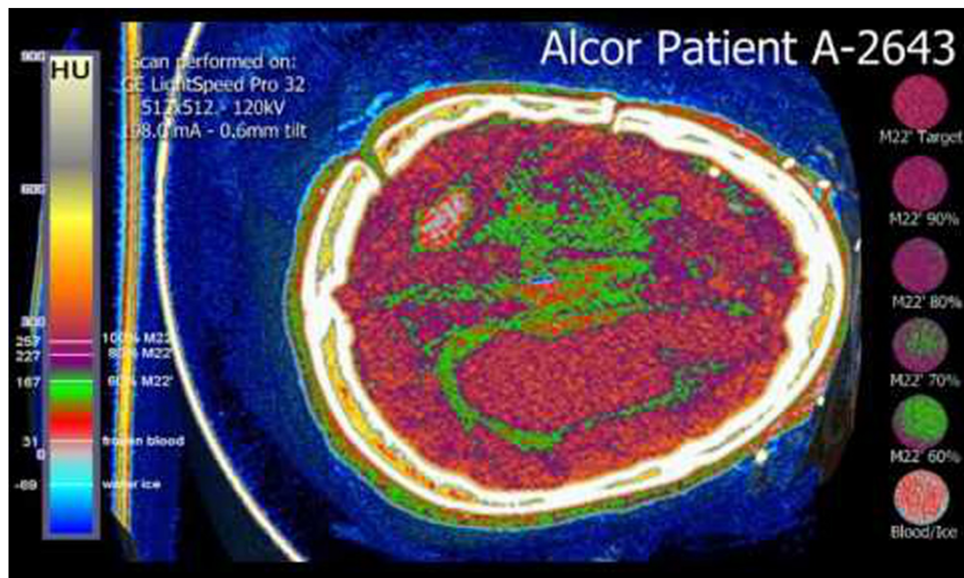

Figure 8. A CT scan of the cryopreserved brain of an Alcor patient.

Cryoprotection of the Ischemic Patient

We define an ischemic patient as one who has sustained periods of normothermic and/or cold ischemia after cardiac arrest, long enough to affect the quality and outcomes of cryoprotection. These different outcomes can range from minor edema and blood brain barrier breakdown to a general inability to do cryoprotection at all. In such cases prolonged ischemia leaves no other option but “straight freezing” without cryoprotection, causing damage in addition to ischemia-induced ultrastructure damage.

The secondary effects of ischemia on cryoprotective perfusion can be divided into the development of edema and perfusion impairment. While these phenomena can be distinctly distinguished it should be kept in mind that (interstitial) edema can narrow the lumen of vessels and obstruct or re-direct perfusion, which in turn can lead to regional differences in cryoprotectant uptake.

One of the earliest signs that ischemia is affecting cryoprotective perfusion is a change in the permeability of blood vessels. In a rat model, blood brain barrier breakdown appeared to be complete after 1 hour of

normothermic ischemia, as evidenced by lack of dehydration of the brain and visual signs of brain edema after completion of perfusion. In a cold ischemia model, breakdown of the blood brain barrier was observed after 24 hours of cold ischemia, regardless of whether the blood was washed out or not. Weight gain after ischemia occurs in a temperature- and time-dependent manner. In fact, in laboratory experiments, whole body weight loss is often observed after completion of cryoprotective perfusion. As the duration of ischemia progresses, this weight loss is not observed and a patient can gain up to 50% in weight in cases with extensive normothermic and cold ischemia. Edema can often be observed in the face and the abdomen seems particularly susceptible to edema, especially when DMSO-based perfusates are used. While cryopreservation without ice formation may still be possible after 48 hours of bloodless cold ischemia (if blood was replaced with MHP-2 blood washout solution prior to the ischemic period), the extensive swelling associated with ischemia cannot be mitigated with any kind of cold organ preservation solution currently known.

Ischemia-induced perfusion impairment is a multi-factorial phenomenon and vascular leaking, interstitial edema, brain swelling, blood coagulation, and red blood cell aggregation combine to produce heterogeneous and incomplete perfusion of the brain. In extreme cases, most parts of the brain will freeze despite cryoprotection procedures. In cases with extensive ischemia longer perfusion times should be expected and it will take longer for venous effluent to reach the target concentration of the vitrification solution. In some cases the refractive index of the venous perfusate fails to further increase at all. In ischemic cases reaching target concentration does not necessarily mean that that all areas of the brain (or body) have received enough cryoprotectant to prevent freezing. Other phenomena that should be expected in the perfusion of the ischemic patient include extensive fluids leaking from the heart and lungs, poor venous return (particularly in whole body cases), ascites, and filling of intestines with perfusate further exacerbating abdominal swelling of whole body patients.

Pharmaceutical interventions to prevent these ischemia-induced challenges are limited. The combination of sodium citrate and heparin have allowed ice-free cryopreservation in the rat model after up to 2 hours of

normothermic ischemia but these benefits disappear if these medications are not administered within 30 minutes of circulatory arrest.

There is some preliminary evidence from the scientific literature on organ preservation and data from cryonics-associated labs that adding streptokinase to the washout solution prior to the start of cryoprotective perfusion could improve perfusion after cold ischemia. High molecular weight polymers (colloids) may be effective to retain perfusate in the vessels in cases where ischemia exposure is limited. When the ischemic period is longer than 1 hour of normothermic ischemia or 24 hours of cold ischemia, swelling will happen nonetheless.

In patients with extensive ischemia it may be especially important to limit perfusion pressures. In the rat model cryoprotection of the brain was improved when maximum arterial pressures were lowered to 80 mmHg. Other cryonics-associated labs have also observed improved outcomes from lowering perfusion pressure. Other approaches that have been tested include the use of hyper-viscous carrier solutions and aggressive ramping to target concentration.

It is currently not possible to give hard criteria for when to abandon cryoprotection in ischemic patients. This determination is also complicated by the fact that ischemic changes in the patient often start prior to pronouncement of legal death. During stabilization, interruptions in procedures, suboptimal CPS, and slow cooling can also produce some degree of ischemia. Another complicating factor is that we have little understanding of the (ultrastructural) effects of the longer perfusion times that are associated with cryoprotection of the ischemic patient.

Based on available case reports, practice, and research cryoprotection still outweighs the adverse of effects of ischemia up to 48 hours of cold (bloodless) ischemia if cryoprotection is possible. Ice-free cryopreservation of the ischemic brain is possible after at least 1 hour of normothermic ischemia, as evidenced by rat and porcine experiments.

A special subset of ischemic cases concerns cryoprotective perfusion of a patient that has sustained an ischemic or hemorrhagic stroke as the cause of death. While cryonics organizations have cryopreserved a number of patients who suffered lethal strokes, no systematic treatment has been written how to

deal with such cases. Some questions that need to be addressed include: Should cryoprotective perfusion be attempted in patients who sustained a (massive) hemorrhagic stroke? Should anti-coagulants and fibrinolytics be administered to either ischemic or hemorrhagic stroke patients? What perfusion pressures should be used in patients who have sustained a stroke? Do hyperosmolar solutions restore circulation to areas of the brain that sustained a stroke? What role can CT scans play in the perfusion of such patients? These are not questions that can be addressed in this manual but we identify these issues here as an important research and clinical topics.

Field Cryoprotection

Two of the most important variables affecting the successful preservation of a patient are time and temperature. They are clearly related. If the time between pronouncement of legal death and the start of cryoprotection is minimized we can place the patient in long-term cryostasis without incurring unnecessary cold ischemic injury. Not surprisingly, it has occurred to a number of people in the cryonics field that the quality of care could be improved if we eliminate the prolonged cold ischemic time that is typically associated with remote cryonics cases (i.e. Alcor cases remote from Scottsdale, Arizona). In this section we will outline potential protocols and challenges concerning field cryoprotection.

Field cryoprotection is the replacement of blood and tissue water by solutions of cryoprotective agents (CPAs) near the location of legal death, followed by prompt cooling to dry ice (–79 degrees C) or lower temperatures at the same remote location. If a temperature cold enough to achieve a solid state is attained (approximately –130 degrees C), the procedure could be called field cryopreservation.

Rationale of Field Cryoprotection

To understand the rationale and challenges associated with the idea of field cryoprotection it is useful to briefly describe the current procedure for remote cases.

When an Alcor member is considered terminal and close to legal death, a standby team should be deployed to the bedside of the patient. For cases outside of Arizona, in the continental United States, the team may be provided by an independent contractor such as Suspended Animation, Inc., and may include a surgeon and clinical perfusionist.

Upon pronouncement of legal death the team begins chest compressions and rapid cooling in an ice bath, and administers a series of medications to mitigate ischemia. If a qualified surgeon is part of the team, or of a cooperating mortician is considered sufficiently competent, an additional procedure is to perform a field washout in which blood is replaced by a cold (but not freezing) organ preservation solution. This procedure is described in Section 16 of this book, discussing remote blood washout.

The three most important objectives of the washout are:

- Increase the cooling rate
- Remove the blood and risk of coagulation and cold agglutination
- Protect the patient against the effects of cold ischemia by introducing an organ preservation solution.

The patient is then shipped on water ice to the cryonics facility for cryoprotective perfusion and long-term care.

Between the end of blood washout and the start of cryoprotective perfusion the patient is basically experiencing a prolonged period of cold ischemia, the duration of which is dependent on variables such as the availability of air transport to the cryonics facility. While experimental evidence at a number of cryonics-associated research labs indicates that remote blood washout is superior to leaving the blood in the patient, it should be evident that prolonged cold ischemia is not beneficial to the patient and could be completely eliminated when there is a smooth transition from stabilization to cryoprotective perfusion. For example, blood substitution with a static organ preservation solution can keep the brain viable (able to spontaneously resume function upon reperfusion with blood) for about 6 hours in the most optimistic projections.

Proposed benefits of field cryoprotection include:

- One single deployment required for both stabilization and cryoprotection
- Elimination (or minimization) of cold ischemia
- One surgical procedure required
- A reduction of total procedure time

Terminology, Historical Background, and Research

In the context of this article, field cryoprotection is defined as the procedure of conducting cryoprotective perfusion at a location remote from the cryonics facility followed by transport of the patient on dry ice for further cryogenic cooldown and long term care at the cryonics facility.

Field cryoprotection is not necessarily the same thing as field vitrification, which would require cryogenic cooling on-site and shipping at around –130 degrees Celsius (below the glass transition temperature of the vitrification agent) or –196 degrees Celsius (liquid nitrogen temperature). While it is not impossible to ship the patient at such temperatures it would introduce a number of non-trivial technological and logistical challenges. This would also likely offset any cost reductions associated with conducting cryoprotective perfusion in the field. As will be discussed below, in field cryoprotection the patient is cooled below 0 degrees Celsius after cryoprotective perfusion but not to a temperature where the vitrification agent solidifies into a glass. For this reason the procedure discussed in this article should be named field cryoprotection (or field cryoprotective perfusion) and not field vitrification or field cryopreservation.

The idea of field cryoprotection is not new and various proposals to introduce the technology have been introduced in the past (including proposals for real field vitrification and shipping below the glass transition temperature). In June 1990 Alcor patient A-1239 received a field cryoprotection with glycerol in Australia prior to shipment on dry ice to Alcor in the USA. In addition, on October 23, 2004, the cryonics company Suspended Animation performed a field cryoprotection with glycerol for the American Cryonics Society prior to shipping the patient on dry ice to the Cryonics Institute for

long-term care. The Cryonics Institute also has authorized field cryoprotection for select (international) cases.

There are number of distinct protocol differences between this current implementation of field cryoprotection and cryoprotection at the Alcor main facility. These protocol differences are not intrinsic to either field cryoprotection or facility cryoprotection however. By historical standards, today's field cryoprotection protocols by Alcor are often more sophisticated than older facility cryoprotection protocols and even contemporary protocols at other cryonics organizations.

One concern that has often been expressed about field cryoprotection is that shipping the patient at dry ice temperature after introducing the vitrification agent could result in ice formation en-route to the cryonics facility. While this concern cannot be completely eliminated, especially when ischemic injury compromises cryoprotectant perfusion, independent results from at least three research labs indicate that this issue does not seem to be a problem for CPA solutions currently used for vitrification in cryonics. The cryobiologist Yuri Pichugin stored large volumes of VM-1 (the vitrification agent used by the Cryonics Institute) and cryoprotected cortical rat brain slices at dry ice temperature without observing ice formation after days of storage. Similar results have been observed in other animal models perfused with M22 at 21st Century Medicine. In 2012 Advanced Neural Biosciences collaborated with Alcor to specifically validate Alcor's proposed field cryoprotection protocol in the rat model and again no ice formation was found after up to 48 hours of storing the brains at dry ice temperature prior to further cooling.

These encouraging research results and experience with this protocol in companion animal cases led Alcor to authorize field cryoprotection for overseas cases that otherwise would end up being "straight freeze" cases (i.e., cryopreservation without cryoprotection).

Whole Body Field Cryoprotection

In principle, field cryoprotection can be conducted in both whole body and neuro cases. Whole body field cryoprotection presents a number of distinct challenges. For starters, a lot more cryoprotectant is needed for whole body cases which for most locations would require the shipping of large volumes of

perfusate (>100 liters) to the location where the patient will be cryoprotected. Usually, though, there should be ample time for this in the opinion of the authors because most cases in which field cryoprotection is feasible and productive involve patients with a prolonged agonal “dying” phase which allows the timely shipping of perfusate. Another challenge is that for the already-large perfusate volumes to remain manageable for whole body patients, a closed-circuit perfusion system would likely be needed. Closed-circuit systems are more complex and require more expertise and experience than open-circuit systems currently used for field cryoprotection of neuropatients (or head-only cryoprotection of whole body patients).

An additional complication involves shipping the patient. Because the patient needs to be shipped on dry ice it is crucial that the cryonics organization has a suitably equipped road vehicle or can comply with airline regulations concerning dry ice and potential weight restrictions. Of course, since cold ischemia is basically eliminated during shipment it would also be possible to transport the patient by ground to the cryonics facility (in whole body cases). Shipment of whole body patients on dry ice has been historically dangerous with significant failure rates due to failure of contractors to follow precise shipping instructions, unexpected airline delays and other causes.

While it is sometimes claimed that one major difference between whole body and neuro cryoprotection involves a difference in surgical procedures, this is not necessarily the case. In case a median sternotomy is chosen to cannulate the heart or aorta both neuro and whole body cryoprotective perfusion can be conducted by just making minor adjustments. A more detailed discussion of potential surgical protocols follows.

Whole-Body Field Vitrification

During 2005, various scenarios for whole-body vitrification were discussed in California during meetings attended by personnel from Suspended Animation, Critical Care Research, and 21st Century Medicine. Options included the use of a specially modified freight shipping container, or the purchase and modification of a semitrailer truck. Liquid nitrogen would be required for rapid cooling, as the demands of an electrically-powered system would exceed the capability of a reasonably sized generator. The most promising scenario

required a vehicle or freight container outfitted for procedures to be driven to a location permitting truck parking near the patient, at which point the patient would be moved to the vehicle in a small van. Meanwhile, a third vehicle would bring liquid nitrogen to the parking location.

After examining many ideas for remote whole-body vitrification, the idea was abandoned due to its logistical complexity.

Surgery

There are basically three options for obtaining vascular access in field cryoprotection: femoral cannulation, aortic cannulation, and carotid cannulation.

Femoral Cannulation. In femoral cannulation a "femoral cut down" is performed to cannulate the femoral artery and vein in a single leg to perfuse the patient. One advantage of this approach is that femoral cannulation used to be the preferred approach for remote blood washout and the cannulae can just remain in place for subsequent cryoprotectant perfusion (even in field cryoprotection, stabilization usually benefits from a washout to accelerate cooling and removing the patient's blood). This approach, however, would not constitute an attractive option for neuro cryoprotection because a lot of perfusate is wasted in perfusing the rest of the body. Another potential disadvantage is that in conditions of ischemia-induced edema perfusion of the brain could be suboptimal. In addition, not all patients have a healthy, patent, femoral artery that will ensure good flow. Yet another disadvantage is the large pressure drop that will occur through a narrow femoral arterial cannula, making perfusion pressure monitoring more difficult. The greatest disadvantage of femoral whole body cryoprotectant perfusion is the difficulty of obtaining adequate venous drainage through a narrow femoral venous cannula.

Aortic Cannulation. In a median sternotomy the chest is opened to cannulate the heart or the ascending aorta. This procedure can be used to either perfuse the whole body or, when the descending aorta (and arms) are clamped, to limit perfusion to the upper body. A major advantage of this approach is that a large organ (the heart) or the widest vessel in the body (the aorta) is selected for perfusion which reduces challenges associated with

cannulating patients with no flow (such as collapsed vessels) and ensures good flow. In a very basic version of the procedure, venous cannulation is not necessary and an opening in the right atrium will suffice for venous drainage. A concern about this approach is that too much perfusate is wasted in neuro cases. Median sternotomy used to be the standard surgical approach for both whole body and neuro cases at Alcor prior to going to isolated head perfusion for neuro patients, and as of this writing is the default approach for all cases at the Cryonics Institute. For neuropreservation cases at Alcor's facility, isolated cephalon perfusion is now the preferred method because it allows better venous drainage, better monitoring of brain perfusion (venous effluent from left and right jugulars can be measured separately), lower likelihood of pushing clots into the brain in cases of significant pre-perfusion ischemia, faster surgery, faster cryoprotectant equilibration, and decreased perfusate utilization.

Carotid Cannulation. Carotid cannulation involves cannulating the carotid arteries, and sometimes the vertebral arteries, in the neck of the patient. This procedure is designed to allow cryoprotective perfusion of the head. As such, this surgical approach is used in neuro cases. It is the simplest cannulation to perform. It focuses on the head (brain) of the patient and minimizes required perfusate volumes. Another advantage is that if the cephalon is perfused separately the whole stump of the head can be used for venous drainage. Disadvantages include the lack of an easy "downstream" fall-back option in case errors are made or the vessels are too fragile or damaged for perfusion. There is also the issue that a determination would need to be made about whether a patient has an intact Circle of Willis. Without this, the vertebral arteries would need to be cannulated, too, for complete perfusion of the brain. When the Circle of Willis is intact, and the head is not separated to allow occlusion of the vertebrals, some perfusion pressure loss can be expected in the Circle of Willis as arterial perfusate leaks from the Circle into the vertebral arteries and into the rest of the body.

One argument against the carotid approach is that unless cephalic isolation is used as an approach for cryoprotection, washout will also need to be restricted to the head unless the team performs two separate cannulations. This may introduce temperature differences between the head and the rest of

the body. In the authors' opinion, the strongest argument against the carotid approach is that there are limited fall-back options in case of failure. If the heart/aortic approach is used, the field team could decide to terminate efforts to conduct cryoprotectant perfusion and transport the patient to Alcor where professional surgeons can attempt carotid cannulation. Field cryoprotective perfusion should allow for a back-up plan in case of failure, which the carotid approach does not permit. The heart / aortic approach also has the advantage that it permits both neuro and whole body cryoprotection.

Protocol

Designing a protocol for field cryoprotection presents five challenges:

1. Ensuring a gradual introduction of the vitrification agent (CPA) to reduce osmotic injury to the cells. When a patient is cryoprotected at the main Alcor facility this goal is achieved by gradually mixing the "carrier solution" with the cryoprotectant in a recirculating reservoir and terminating perfusion when the desired terminal concentration of the agent has been consistently observed in venous fluid. In field cryoprotection such a recirculating setup would be complicated and current field cryoprotection protocols involve introducing a series of bags with increasing concentrations of the vitrification agent. Terminologically, the current field cryoprotection protocol is "open circuit" perfusion in which venous flow is discarded, while Alcor's facility protocol is "closed circuit" perfusion in which venous flow is recirculated. In Alcor's established protocol for field cryoprotection of the head through carotids (called "Field Neuro Cryoprotection" or FNCP), bags can be (and are) overlapped using a "teeter-totter" which blurs the jump between steps, further smoothing the introduction of different concentrations.

2. Maintaining a cryoprotectant concentration ramp and peak concentration plateau that is long enough to be comparable to what is achieved in cryonics facilities utilizing closed-circuit perfusion. This is difficult with open-circuit perfusion while keeping the perfusate volumes manageable, especially for whole body patients.

3. Temperature control. At the Alcor main facility cryoprotective perfusion is started at +3 degrees Celsius and lowered to about -3 degrees Celsius for the final half of the procedure to mitigate the cryoprotectant toxicity associated with higher concentrations. In field cryoprotection, subzero perfusion presents a bigger challenge and would require an enclosure with circulating nitrogen gas and running the perfusate through a heat exchanger (HEX) capable of reducing the temperature below the freezing point of water. Alcor's current field neuro cryoprotection protocol involves keeping the temperature of the patient as close to 0 degrees Celsius as possible by surrounding the patient with ice packs, and adding antifreeze to an ice water bath to facilitate lowering of the perfusate temperature below 0 degrees Celsius.
4. Monitoring the refractive index (or BRIX reading) of the vitrification agent as the concentration increases. At the Alcor main facility the concentration of the vitrification agent is continuously monitored in the perfusion lines to observe trends. Decisions as to whether to continue or stop perfusion are made using a benchtop refractometer. In field cryoprotection continuous inline monitoring of concentration of the vitrification agent would be challenging and the current protocol requires the use of a handheld digital refractometer to make frequent refractive index (or BRIX) readings to observe trends and to decide whether to continue or end perfusion.
5. Controlling flow rate and pressure. There are two options for controlling flow of the perfusate in the patient: a pump or a hanging bag system. The major advantage of using a pump is that it provides precise control over flow rates and pressure. The advantage of a hanging bag system is that no priming of the pump and other associated challenges need to be performed. No power supply for a pump is required, and pressure spikes are limited by the height of the bags. In reality, the choice of either a pump or a bag will greatly depend on the degree of expertise and experience in the field.

The current Alcor protocol for field cryoprotection under discussion employs an 8-step bag system (including washout with B1 carrier solution), as shown in Table 4.

Bag	nM22 Concentration	Refractive Index (BRIX)
Bag 1	0%	9.8
Bag 2	5%	11.81
Bag 3	8%	13.12
Bag 4	14%	15.31
Bag 5	23%	18.94
Bag 6	50%	29.85
Bag 7	50%	29.85
Bag 8	104%	51.50

Table 18-4. The system of stepped field perfusion used by Alcor requires eight bags containing concentrations of perfusate as shown.

The term nM22 denotes a solution make by diluting 125% M22 solutes prepared in LM5 carrier solution with B1 carrier solution to achieve the stated concentrations. The percent concentration scale is not concentration of solutes, but percent full concentration of M22, which has defined solute concentrations. 100% M22 or 100% nM22 is also sometimes called 100% CNV (concentration needed to vitrify) to express the idea that tissue is ideally to reach full M22 solute concentration before stopping perfusion and attempting vitrification by cooling. The endpoint for perfusion in this protocol has been measurement of jugular effluent of nM22 over 49.9 BRIX refractive index (100% CNV) for over 30 minutes. This protocol ensures a concentration necessary to vitrify (CNV) in the cells without prolonged exposure to even higher concentrations.

Two Visions of Field Cryoprotection

While Alcor has authorized field cryoprotection for overseas cases, during 2017 a debate was continuing about the desirability of introducing field cryoprotection for most Alcor members who are pronounced legally dead in

the United States and Canada. Issues that have been discussed include scientific, technological, and financial concerns.

Alcor's facility cryoprotection procedures are designed to closely replicate laboratory research protocols that have shown published efficacy for brain cryopreservation. They are based on established principles of organ cryopreservation for minimizing osmotic and cryoprotectant injury while eliminating ice formation. To what extent do simplified and briefer open-circuit field cryoprotection protocols compromise cryopreservation quality?

Alcor's facility infrastructure includes computerized control and recording of multiple perfusion parameters, and personnel for observation and note-taking. To what extent will field cryoprotection quality suffer because of decreased perfusion parameter control, decreased data recording, and resulting decreased quality control feedback?

Can a patient be shipped on dry ice without risking ice formation during transport to the cryonics facility?

What is the easiest and safest surgical approach? How many concentrations of the vitrification agent need to be used? Can we lower the cost of our procedures by embracing field cryoprotection?

Perhaps the most difficult question of all is: At what distance and transport time from Alcor do the disadvantages of current field cryoprotection procedures (especially no cryoprotection for the body of whole body patients) become outweighed by the advantages of avoiding long transport times at 0 degrees C?

There are some who worry that simplified field cryoprotection procedures with limited monitoring are driven by a desire to reduce costs, complexity and oversight rather than strict improvement of care and cryopreservation outcome. Yet clearly there are distances for which even the simplest field cryoprotection protocols are beneficial, such as locations with multiday transport times.

A sensible approach to evaluate these issues is to ask whether the primary aim of field cryoprotection is improvement of patient care or simply reduction of cost. While it is indisputable that the elimination of two separate deployments can lower the costs associated with Alcor's procedures (assuming field cryoprotection protocols that are deliverable by current

standby teams), these different perspectives can lead to different views on how to conduct field cryoprotection.

If field cryoprotection is primarily advocated as a means to improve patient care the most likely implementation for Alcor is to request its standby provider (currently Suspended Animation for non-local cases) to add field cryoprotection to its washout procedure. While it would be simplistic to argue that this would just involve simply adding a few bags of perfusate to the washout procedure, it should be recognized that an organization that employs professional surgeons to establish surgical access and professional perfusionists for running the pumps should be able to perform this procedure without formidable challenges. If the aim, on the other hand, is to just reduce cost and involve Alcor staff and volunteers in field cryoprotection, the most conservative surgical protocols and cryoprotection protocols would need to be followed to reduce errors.

In our opinion, it is not possible to have a sensible discussion about the nature and scope of field cryoprotection without asking who is going to perform it. If Alcor entrusts the conduct of remote blood washout to qualified independent contractors, then concerns about the absence of relevant surgical and perfusion skills may not be all that relevant. If field cryoprotection is seen as a substitute for these contracts, however, Alcor would be making a challenging leap into the unknown.

Alternatives for Field Cryoprotection

The only credible alternative for field cryoprotection would be to validate and introduce organ preservation solutions aimed at securing viability of the brain, or at least perfusability of the brain, at above-freezing temperatures for much longer than is possible with Alcor's current organ preservation solution (MHP-2). In essence, this would require the design and successful validation of "brain preservation solutions" that can preserve cerebral viability for up to 24 or 48 hours of ischemia at a temperature barely above 0 degrees Celsius. While 21st Century Medicine has made a number of breakthroughs in organ preservation solution design that enable viability of the brain for much longer periods than is possible with MHP-2, these protocols require either continuous or intermittent perfusion of the patient (or the patient's brain) en route to the

main cryonics facility. This fact by itself necessitates ground transport of the patient under supervision of qualified staff, which in some cases could involve many days.

Another concern with continuous perfusion protocols is that there is little information available on their effects in cases where warm ischemia has occurred. Prior research in the art would indicate that continuous perfusion in an ischemic patient, especially in a whole body patient, will produce severe edema over a long period of time. This edema could prevent any meaningful cryoprotective perfusion at the main facility, defeating the main objective of blood substitution.

In conclusion, the most basic question is rather straightforward. If cryoprotectant perfusion can be done competently in the field, affordably, and without much sacrifice in quality, and if it can allow much better outcomes in terms of elimination of cold ischemia and ice formation, why continue the tradition of transport on ice after remote washout? In the long term, there is no theoretical reason that everything currently done in Alcor's facility operating room couldn't be done at remote locations. The challenges of designing equipment, training personnel, and funding the operation would be significant, but we may still debate how sophisticated field cryoprotection really needs to be to be beneficial at various distances.

The frequency with which personnel perform procedures is a strong determinant of the competency with which the procedures are performed. The importance of frequent performance increases with greater complexity of procedures and equipment. Even if all cryonics cases over the whole world were performed at a single facility, the number of cases at the facility would still be small by normal standards of medical practice proficiency. In the editor's opinion, for complex cryoprotectant perfusion procedures to be competently performed at decentralized locations by traveling experienced professionals will require growth of cryonics popularity substantially beyond what exists today (2019).