HISTORY OF DMSO AND GLYCEROL IN CRYONICS

By Mike Darwin

In The Beginning: Vitrification or Freezing?

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

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

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

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

The First Human Cryopreservation Protocol

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

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

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

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

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

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

Photo by Ted Kraver

Figure 1. Robert Ettinger *(foreground) demonstrates use of the Iron Heart, a mechanical chest compressor.*

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

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

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

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

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

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

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

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

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

Figure 2. Dr. Dante Brunol, 1967

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

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

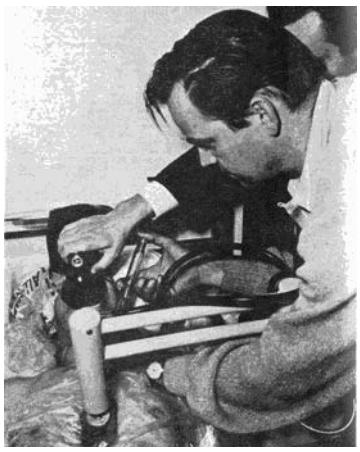


Photo by Robert Prehoda

Figure 3. Robert F. Nelson *(foreground) with Dante Brunol attending Bedford on the night of 12 January, 1967. The Iron Heart is a prop for the photographs; it was not used during Bedford's cryopreservation.*

Glycerol and the Cryonics Society of New York

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

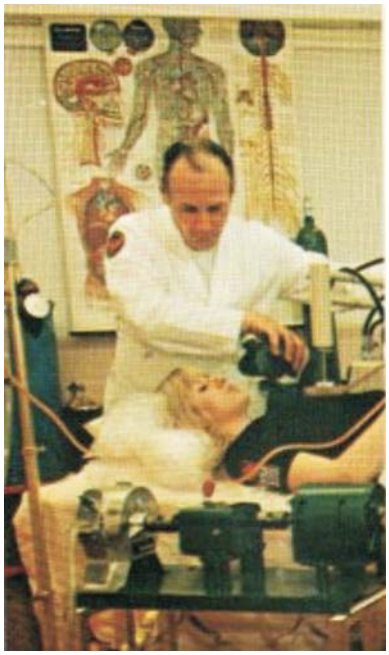


Figure 4. Amtec 209 Roller Pump. *The Amtec pump is in the foreground (green) and the Zero-Max controller is the rectangular gear box with the red lever between the motor on the right, and the pump head on the left.*

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

The bubble trap was designed by a physician associated with CSNY at that time, Dr.

Jane Enzman, daughter of the maverick physicist and engineer Dr. Robert Duncan Enzman.

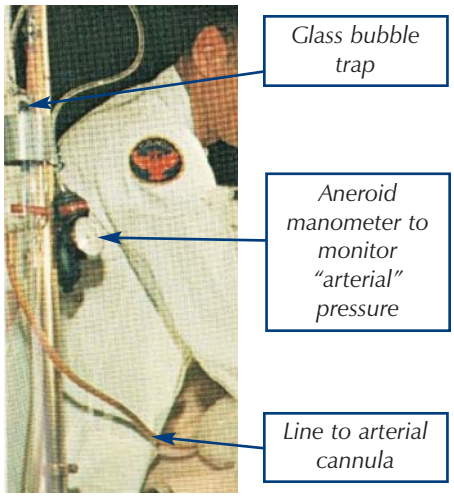


Figure 5. Bubble Trap. *The Bubble Trap is a custom-made glass bubble trap, with an attached aneroid manometer, to monitor perfusion pressure.*

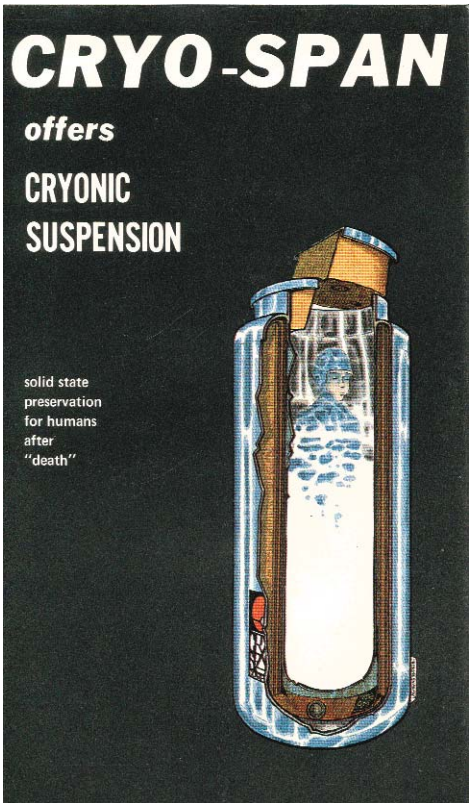


Figure 6. Cover of the Cryo-Span brochure *(circa 1971)*

Unfortunately, this set-up was never used on a patient. CSNY used a Porti-Boy embalming machine.

Figure 7: Porti-Boy

Paul Segall modified the Brunol protocol in a number of unfortunate ways. Segall eliminated any attempt at maintaining post-arrest circulation writing, *"No attempt is made to maintain circulation of the blood for the following reason. It has been observed that if the blood flow falls under 70 mm Hg for more than 5 minutes there is irreversible damage (by today's standards) to the cerebral brain centers. Evidence has shown that perhaps this [is] due to the blockage of the microcirculation of the brain (the capillaries become clogged because of the formation of blood clots). In all likelihood, artificial circulation after death could not be started fast enough to reach the cerebral centers (9)."*

Figure 8. Dr. Paul Segall, 1978

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

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

Learning the Hard Way

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

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

as good external cooling. While edema was slower to develop, it nevertheless occurred, and again resulted in failure of venous return (14).

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

Figure 9. Jerry Leaf, 1978

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

As of 1977, I had decided that DMSO was unacceptable, because of the consistent problems with edema observed, and its documented destructive effects on the vascular endothelium of kidneys being perfused for

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

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

Figure 11. Rabbit Brain Experiments 1979

was used with a perfusion time (combined head and trunk) of 157 minutes. The final cranial (right internal jugular) effluent glycerol concentration was ~4.0 M glycerol.

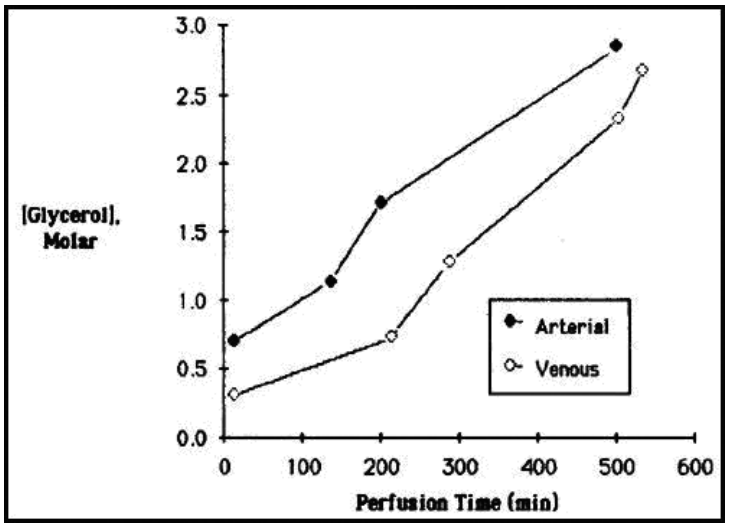
Despite the comparatively large volumes of perfusate used this patient did not develop edema. (Note: until 1981 this volume of cryoprotective perfusate would have been considered large.) In the winter of 1977, under the auspices of the Institute for Advanced Biological Studies in Indianapolis, IN, I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

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

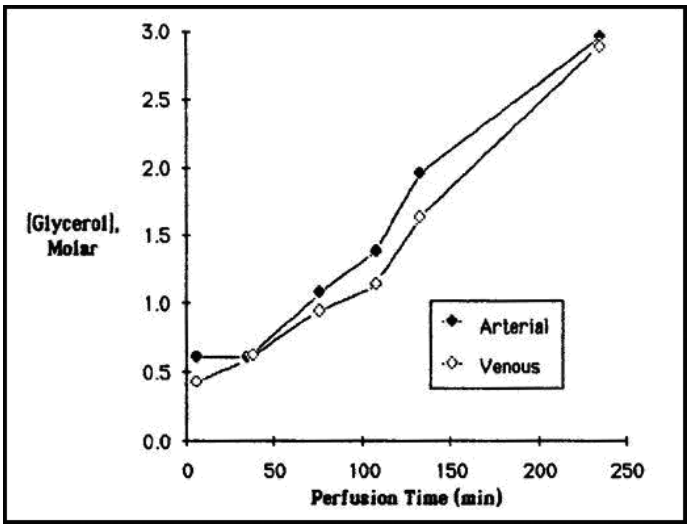
5% glycerol perfusate, 25 liters
10% glycerol perfusate, 10 liters
15% glycerol perfusate, 10 liters
20% glycerol perfusate, 10 liters
25% glycerol perfusate, 10 liters
50% glycerol perfusate, 15 liters

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

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



Graph A. SP1 Glycerol Concentration vs. Perfusion Time



Graph B. SP2 Glycerol Concentration vs. Perfusion Time.

ed by BioPreservation in 1995 (22), terminal tissue glycerol concentration was increased to 7.5 M (the maximum concentration possible to perfuse due to viscosity constraints).

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

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

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