

Cryoprotectant Toxicity

by Ben Best

In cryonics we seek to preserve humans and pets at cryogenic temperatures while minimizing damage associated with our preservation methods. Prior to cooling to cryogenic temperatures (temperatures below -100°C) we perfuse with cryoprotectant solutions in the hope that we can vitrify tissue with these "anti-freeze" cryoprotectants rather than allow ice to damage the tissues. **Vitrification** means the complete elimination of ice by the use of cryoprotectants and ice-blocking agents.

All cryoprotectants when used in concentrations adequate to vitrify have some toxicity (as measured by their negative effect on cell function and cell survival). For best preservation we would want to not only eliminate ice formation using cryoprotectants, but eliminate toxicity from cryoprotectants. Reversible cryopreservation ("suspended animation") could be achieved if it were possible to find cryoprotectant mixtures that were sufficiently powerful and non-viscous to vitrify tissues without having toxic effects. Experimentation and speculation to date has not provided a very clear idea of the molecular mechanisms of cryoprotectant toxicity and how to prevent it. Nor is there a good understanding of the molecular mechanisms by which cryoprotectants eliminate ice formation.

If there is a Manhattan Project in cryonics it should be devoted to the problem of cryoprotectant toxicity. Resources allocated to nanotechnology, whole body vitrification, intermediate temperature storage, etc. may be of some value, but they can also be a distraction from the most important research goal for cryonics -- elimination of cryoprotectant toxicity. It would be better to concentrate intellectual and financial resources to the central issue of cryopreservation by vitrification.

Vitrification mixtures of low viscosity that are non-toxic enough to preserve human organs at cryogenic temperatures without loss of viability would attract vast amounts of research investment which would further advance the science and the goals of cryonics. Conventional medicine in hospitals, clinics and universities would understand the lifesaving importance of our goals of reducing ischemic damage by hypothermia, CardioPulmonary Support (CPS) and other means as the first step in cryopreservation. Solving the problem of cryoprotectant toxicity is the key to a breakthrough in the enlisting scientific and financial resources into cryonics practice.

Cryoprotectants are assessed by means of a number of parameters, including permeance, viscosity, toxicity, and concentration needed to vitrify (C_v). C_v is the minimum required concentration of the particular CPA which will vitrify, which is an important quantity to keep in mind because concentrations too much above this minimum result in increased toxicity without increased benefit.

Glycerol was the first CryoProtectant Agent (CPA) to gain widespread use in cryobiology, for cryopreserving red blood cells and sperm. The value of DMSO (DiMethylSulfOxide) as a CPA was discovered not long thereafter. Other polyols, such as ethylene glycol (automobile anti-freeze) and propylene glycol (formerly used to reduce ice formation in ice cream) were later shown to be effective cryoprotectants. Amides are weak cryoprotectants compared to polyols (formamide is too weak to vitrify on its own, but can assist vitrification by other cryoprotectants). Both amides and polyols become stronger cryoprotectants (have lower C_v) by the addition of methyl groups.

High levels of sugars and sugar alcohols are found in many polar plants, insects, fungi, etc. as non-toxic cryoprotectants. The fact that fructose will not crystallize is the reason sucrose is used as table sugar, despite the fact that fructose is cheaper. Monosaccharides can dissolve in cryoprotectant solutions more readily and vitrify at lower concentrations than disaccharides [CRYOBIOLOGY; Kuleshova,LL; 38(2):119-130 (1999)], but because of their capacity for glycation, monosaccharide exposure to protein should be brief and at low temperature. Sugars are more often used as cryoprotectants against freezing and chilling injury rather than for vitrification, with the disaccharide sucrose being more effective than the monosaccharide glucose [CRYOBIOLOGY; Santarius,KA; 20(1):90-99 (1983) and CRYOBIOLOGY; Carpenter,JF; 25(3):244-255 (1988)]. For mint shoot tips, sucrose reduces the toxicity of ethylene glycol and DMSO at 22°C and of glycerol at 0°C [CRYOBIOLOGY; Volk,GM; 52(2):305-308 (2006)].

To the extent that glycation plays a role in enzyme stability and membrane stability associated with freezing damage or chilling injury, trehalose is a superior cryoprotectant to sucrose. Trehalose interacts more strongly with water than does sucrose, at least partly because sucrose forms intramolecular hydrogen bonds [THE JOURNAL OF PHYSICAL CHEMISTRY B; LeBret,A; 109(21):11046-11057 (2005)]. At the phospholipid bilayer of cell membranes trehalose is able to displace water molecules bound to carbonyls, but sucrose is not [[BIOPHYSICAL JOURNAL; Amalfa,F; 78\(5\):2452-2458 \(2000\)](#)]. Trehalose has a higher glass transition temperature (T_g) than sucrose. At 5% water content, T_g for trehalose is about 40°C whereas T_g for sucrose is about 15°C . Addition of 5% trehalose to 10% DMSO solution has been shown to enhance survival of nucleated cord blood cells from 7% to 25%, depending on the cell type [TRANSFUSION; Zhang,XB; 43(2):265-272 (2003)].

Glycerol can cause kidney failure by increased hydrogen peroxide generation [AMERICAN JOURNAL OF PHYSIOLOGY; 257(3 Pt 2):F440-F445 (1989)] and increased nitric oxide release [RENAL FAILURE; 28(2):161-168 (2006)]. Toxic metabolites of ethylene glycol can cause kidney and cardiopulmonary failure [FORENSIC SCIENCE INTERNATIONAL; 155(2-3):179-184 (2005)]. But cryoprotectant toxicity in cryobiology (and cryonics) is not related to long-term, high-temperature systemic effects -- particularly liver breakdown products like ethylene glycol metabolites.

For kidney slices glycerol is generally the least toxic of the of the conventional CryoProtectant Agents (CPAs), which can be ordered by toxicity as:

formamide > propylene glycol > DMSO > ethylene glycol > glycerol

With the exception of formamide, this is the exact order of the vitrifying capability of these CPAs, with glycerol being both the least powerful CPA and the least toxic [CRYOBIOLOGY; 40(2):151-158 (2000)]. Formamide is the most toxic CPA, but has the least glass-forming ability (it cannot vitrify by itself, but can assist vitrification by other cryoprotectants). For human and mouse oocytes, however, DMSO may be more toxic than propylene glycol [HUMAN REPRODUCTION; 8(7):1101-1109 (1993)]. And for human endothelial cells, even at 2-4°C DMSO and ethylene glycol are far more toxic than propylene glycol or 2,3-butanediol [CRYOBIOLOGY; 44(1):24-37 (2002)].

Unlike the **polyols** (glycerol, ethylene glycol, propylene glycol, etc.), the toxicity of DMSO can be reduced by mixing with other CryoProtectant Agents (CPAs). Interestingly, the rank order for heat-release-on-mixing of DMSO with other cryoprotectants is the same as the order of effectiveness of reducing DMSO toxicity [CRYOBIOLOGY; 24(3):196-213 (1987)]:

formamide > ethylene glycol > propylene glycol > N-methyl formamide

probably because associating more strongly with DMSO corresponds with reducing its toxicity. Methylation of polyols increases glass-forming ability while increasing toxicity due to increased hydrogen bonding strength of the hydroxyl groups [JOURNAL OF PHYSICAL CHEMISTRY; 94:6889-6893 (1990)].

Understanding the molecular mechanisms of the toxicity of cryoprotectant agents would be an important step toward finding means of reducing toxicity. Correlations have been found between cryoprotectant toxicity and destabilization of proteins in cell membranes [PHARMAZIE; Ivanov,IT; 56(10):808-809 (2001)]. DMSO perturbs cell membranes, possibly by displacement of water [BIOCHEMICA ET BIOPHYSICA ACTA; Westh,P; 1664(2):217-223 (2004)]. DMSO is more hydrophobic at higher temperature, so there is less DMSO membrane dehydration (toxicity) at lower temperature [[BIOPHYSICAL JOURNAL; Sum,AK; 85\(6\):3636-3645 \(2003\)](#)]. DMSO oxidizes sulfhydryl groups, an effect which would be expected to decrease with lower temperature [BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS; Snow,JT; 64(1):441-447 (1975)]. DMSO, glycerol and especially propylene glycol can all form potentially toxic formaldehyde by non-enzymatic reactions [[HUMAN REPRODUCTION; Karran,G; 11\(12\):2681-2686 \(1996\)](#)]. DMSO, formamide and methanol have been shown to dissolve DNA at high concentrations and temperatures above 20°C [BIOTECHNOLOGY AND BIOENGINEERING; Bonner,G; 68(3):339-344 (2000)]. The enzyme thermolysin is 95% inactivated by a 50% DiMethylFormamide (DMF) at 80°C within 20 minutes, but a 50% DMSO solution only results in 10% inactivation under the same conditions. Addition of 20% glycerol reduces inactivation by DMF to 90% and trehalose reduces the inactivation by 80% [JOURNAL OF BIOTECHNOLOGY; Pazhang,M; 127(1):45-53 (2006)].

Many cryobiologists operate as though different toxicity rules apply to different cells, tissues, organs and organisms -- limiting their focus to their own specialties. A Unified Theory of Cryoprotectant Toxicity must be possible, and is should be the sought in order to have the deepest understanding of mechanisms. Plants, oocytes, and fish embryos are composed of proteins, lipids, carbohydrates and nucleic acids no less than mammalian organs. Studying and understanding a wide variety of organisms and tissues provides the best potential for learning what is general and what is specific about CPA toxicity -- which must ultimately come down to the interactions of CPAs with proteins, lipids, carbohydrates, nucleic acids, and water as well as interactions of CPAs.

Cryoprotectant toxicity could be caused by denaturation of proteins (the **protein denaturation hypothesis**) [CRYOBIOLOGY; 27:401-415 (1990)], although there is evidence that proteins in general are not denatured at the temperatures and cryoprotectant concentrations relevant to vitrification [CRYOBIOLOGY; 27:247-268 (1990)]. The cryoprotectants that vitrify most powerfully are those that hydrogen-bond strongly to water, thereby interfering with the water-to-water hydrogen-bonding that is the basis of ice. But those same cryoprotectants may also hydrogen-bond most strongly to proteins, causing the most unfolding and the most protein (enzyme) denaturation. The least toxic cryoprotectants prevent ice formation by weak hydrogen-bonding, but more importantly by colligative interference with ice formation. The **dehydration damage hypothesis** asserts that toxic cryoprotectants cause dehydration damage by binding to water molecules, thereby preventing the water molecules from properly hydrating proteins and other macromolecules. There is evidence against this explanation. Dehydration damage is only caused by the removal of water which is

bound to proteins. Bound water can allow dehydrated cells with high protein content to vitrify without cryoprotectant. Powerful cryoprotectants toxic at low doses would find plenty of bulk water to hydrogen-bond.

Until recently, there was no means of predicting cryoprotectant toxicity. Producing cryoprotectant cocktails with low toxicity and high vitrification capability was a matter of random trial and error. However, 21st Century Medicine researchers Gregory M. Fahy and Dr. Brian Wowk discovered a metric symbolized as qv^* can be used to quantify toxicity as a function of molecular polar groups at concentration needed to vitrify [CRYOBIOLOGY 48(1):22-35 (2004)]. For

$$q = \frac{M_w}{M_{PG}} = \frac{\text{moles of water}}{\text{moles of polar groups on penetrating cryoprotectant}}$$

the quantity qv^* can be used to control cryoprotectant toxicity -- where qv^* refers to q at a concentration needed to vitrify (C_v , the " v " in qv^*) 5-10 ml of solution at a cooling rate of about 10°C per minute (the "*" in qv^*) down to glass transition temperature (T_g). Moles of polar groups (M_{PG}) are arrived at by simple counting. For example, M_{PG} of glycerol is 3 hydroxyl groups, M_{PG} of ethylene glycol is 2 hydroxyl groups, and M_{PG} of DMSO is 1 (the sulfinyl group of the sulfoxide). Toxicity and glass-forming ability varies linearly with qv^* . For most cryoprotectants, qv^* is in a range between 2 and 4, but DMSO has a qv^* of about 6. (Note that v and $*$ define the standard conditions of q -- they are not multiplied times q .) Minimizing qv^* results in vitrification with minimum toxicity.

21CM researcher Dr. GM Fahy's most effective and least toxic vitrification solution for nearly a decade was **VS4** (Vitrification Solution 4), composed of 14% w/v DMSO, 14% w/v formamide, 11% w/v propylene glycol and 10% w/v colloid [TRANSPLANTATION 70(1):51-57 (2000)] -- although this mixture can only vitrify at 1,000 atmospheres of pressure. Increasing propylene glycol to about 17% w/v allowed vitrification at 1 Atmosphere pressure, so the modified formula was called **VS41A**. By replacing propylene glycol with ethylene glycol, however, the 21 CM researchers significantly increased viability -- and the new cocktail is called **Veg** -- ie, VS41A with ethylene glycol.

Concentration of penetrating cryoprotectant (and hence toxicity) can also be reduced by use of nonpenetrating cryoprotectants such as large molecular-weight polymer (eg, polyvinylpyrrolidone or polyethylene glycol) or sucrose. Nonpenetrating cryoprotectants are too large to diffuse into cells, but they assist with vitrification of water (and inhibition of devitrification) in the extracellular space. Less cryoprotectant is needed inside cells than in the extracellular space because of dehydration (which drives water from cells into the extracellular space) and because cells naturally contain proteins that enhance vitrification. While cryoprotectants slow ice-crystal growth and formation, ice blockers act specifically against the formation of the ice nuclei which are necessary for freezing to begin. Arctic fish use ice-blocking proteins to keep the freezing temperature of their bodies at or below -2.2°C , which is below the freezing temperature of seawater (-1.7°C). The researchers report optimizing cryoprotectant vitrification while minimizing toxicity by a judicious use of (1) penetrating cryoprotectants (selection based on qv^*) (2) non-penetrating cryoprotectants and (3) ice-blockers.

By adding ice-blockers and a judicious mixture of other CPAs to **Veg** the 21CM researchers developed a new vitrification solution called **M22** (so-named because of the intention to introduce this vitrification cocktail to a biological specimen at **Minus 22°C**). M22 has been used to vitrify a rabbit kidney at -135°C , which was subsequently rewarmed, transplanted into a rabbit and sustained the life of the rabbit as the sole functioning kidney.

In general, cryoprotectant toxicities are lower at lower temperature, and may even become negligible if the cryoprotectants can be introduced at a low enough temperature. In perfusing the brain at the Cryonics Institute, ethylene glycol is introduced first, which can allow subzero temperature to be achieved (without freezing) before a DMSO/ethylene glycol mixture is introduced. The ethylene glycol also reduces the toxicity of DMSO. Unfortunately, cryoprotectants become increasingly viscous at low temperature, reducing their capacity to diffuse into tissues. So there is a trade-off of reduced toxicity and the requirement for greater exposure times to achieve tissue saturation at low temperature.

Cryoprotectant toxicity is of vast importance for cryonics. Although enzymes should be easy to replace, and denatured membrane proteins may not cause so much structural damage, the goal of cryonics-directed cryobiological research should be to cause the least amount of damage possible. Lessening damage lessens the chance of irreparable damage (ie, destruction). And the potential for reversible cryopreservation of whole organs will not only bring credibility to cryonics, but will greatly increase research and save lives through organ transplantation.