HOW CRYOPROTECTANTS WORK

By Brian Wowk, Ph.D.

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

How Freezing Injures Cells

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

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

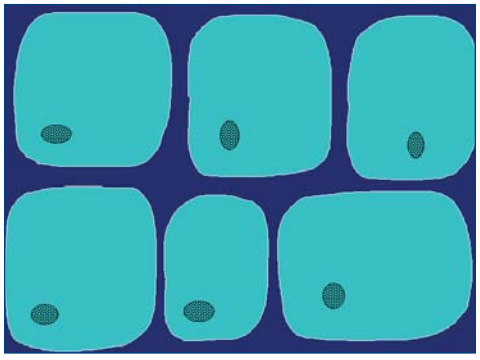

Figure 1A. Cells before freezing.

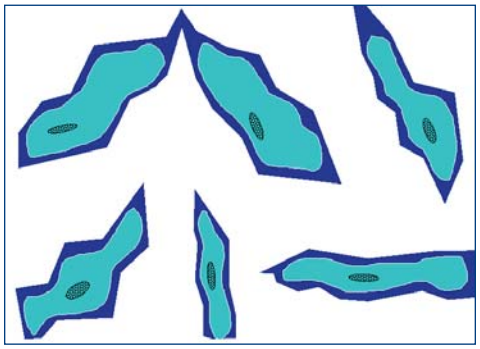

Figure 1B. Cells after freezing. *Cells are squashed between ice crystals and exposed to lethal concentrations of salt. Contrary to popular belief, slow cooling causes water to freeze outside cells, not inside cells. Cells are dehydrated by the growing concentration of salt in the unfrozen liquid around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

How Cryoprotectants Protect Cells

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

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

Properties of Cryoprotectants

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

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

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

Vitrification as an Alternative to Freezing

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

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

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

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

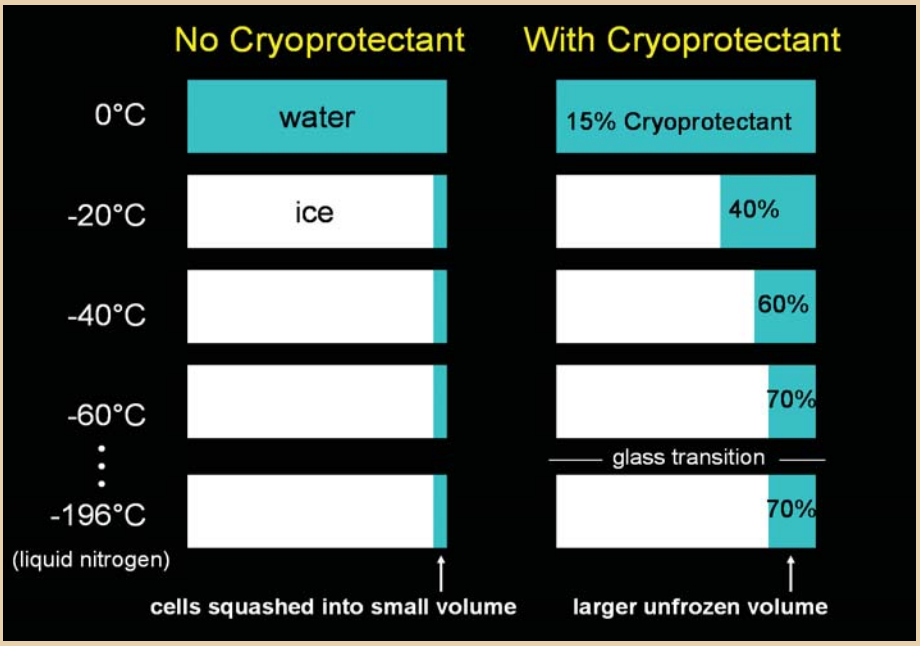

Figure 2. Water when frozen without and with added cryoprotectant. *Without cryoprotectant, almost the entire water volume freezes during cooling. Only salts and other dissolved molecules prevent water from freezing completely. With cryoprotectant, the percentage of cryoprotectant present in solution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

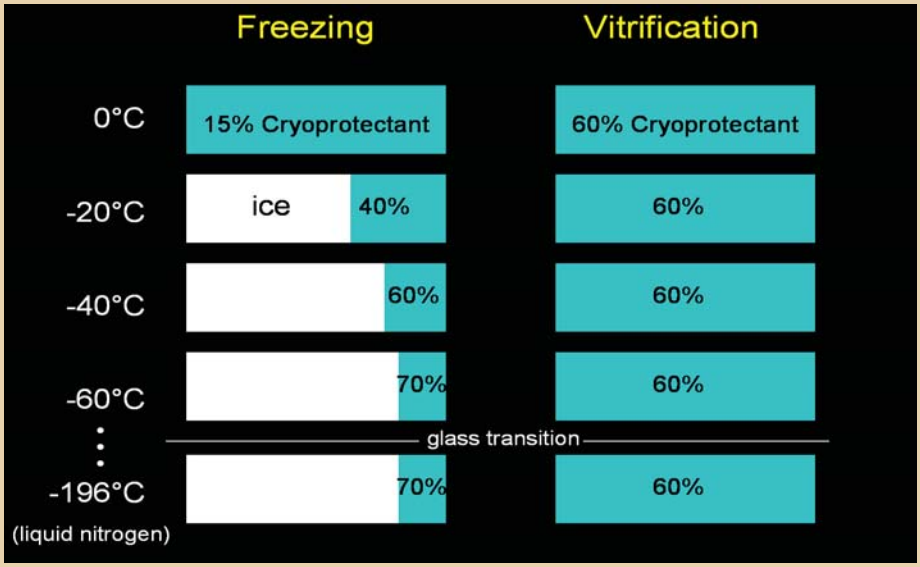

Figure 3. Freezing vs. vitrification. *Vitrification loads cells and tissue with a high concentration of cryoprotectant at the very beginning. Cooling quickly then allows the entire volume of tissue to become a glassy solid, or "vitrify", without any freezing at all.*

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

Cryoprotectant Toxicity

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

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

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

Components of Cryopreservation Solutions

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

Carrier Solution

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

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

Penetrating Cryoprotectants

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

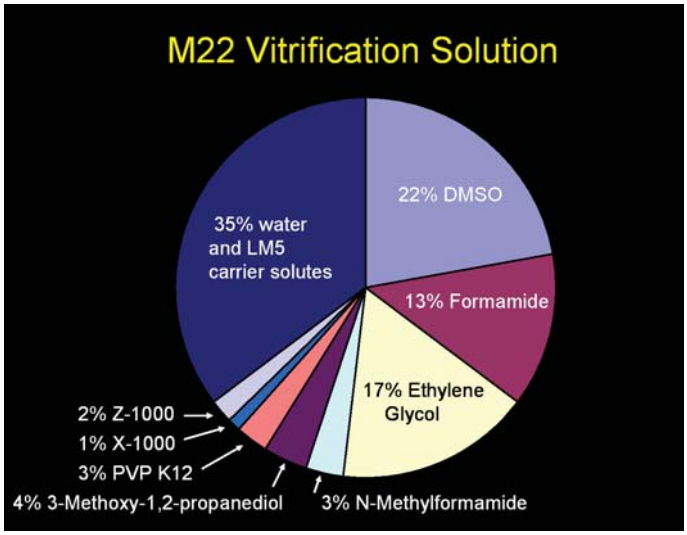

Figure 4. Composition of M22 vitrification solution. *All ingredients are penetrating cryoprotectants, except for LM5 carrier solutes, Z-1000 and X-1000 ice blockers, and PVP K12 polymer. M22 is a "sixth generation" vitrification solution, incorporating two decades of progress in the development of vitrification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

Non-penetrating Cryoprotectants (optional ingredient)

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

Ice Blockers (optional ingredient)

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

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

How Cryoprotectants are Used

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

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

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

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

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

Special Considerations for Organs

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

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

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

Six Generations of Vitrification Solutions

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

Generation 1

The simplest vitrification solutions are single cryoprotectants in carrier solution.

Generation 2

It was discovered that higher total cryoprotectant concentrations with acceptable toxicity

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

Generation 3

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

Generation 4

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

Generation 5

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

Generation 6

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

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

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

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

References

1. C. Polge, A.U. Smith, A.S. Parks, Revival of spermatozoa after vitrification and dehydration at low temperature, Nature 164 (1949) 666.
2. A.M. Karow, Cryoprotectants—a new class of drugs, J. Pharm. Pharmac 21 (1969) 209-223.
3. M. Fahy, D.R. MacFarlane, C.A. Angell, H.T. Meryman, Vitrification as an approach to cryopreservation, Cryobiology 21 (1984) 407-426.
4. G.M. Fahy, The Relevance of Cryoprotectant "Toxicity" to Cryobiology, Cryobiology 23 (1986) 1-13.
5. G.M. Fahy, Cryoprotectant Toxicity and Cryoprotectant Toxicity Reduction: In Search of Molecular Mechanisms, Cryobiology 27 (1990) 247-268.
6. G.M. Fahy, B. Wowk, J. Wu, S. Paynter, Improved vitrification solutions based on the predictability of vitrification solution toxicity, Cryobiology 48 (2004) 22-35.
7. I.B. Bakaltcheva, C.O. Odeyale, B.J. Spargo, Effects of alkanols, alkanediols and glycerol on red blood cell shape and hemolysis, Biochem Biophys Acta 1280 (1996) 73-80.
8. B. Wowk, E. Leitl, C.M. Rasch, N. Mesbah-Karimi, S.B. Harris, G.M. Fahy, Vitrification enhancement by synthetic ice blocking agents, Cryobiology 40 (2000) 228-236.
9. B. Wowk, G.M. Fahy, Inhibition of bacterial ice nucleation by polyglycerol polymers, Cryobiology 44 (2002) 14-23.
10. G.M. Fahy, unpublished.
11. G.M. Fahy, B. Wowk, J. Wu, J. Phan, C. Rasch, A. Chang, E. Zendejas, Cryopreservation of organs by vitrification: perspectives and recent advances, Cryobiology 48 (2004) 157-178.
12. K.G. Brockbank, Y.C. Song, Morphological analyses of ice-free and frozen cryopreserved heart valve explants, J. Heart Valve Dis. 13 (2004) 297-301.
13. Y.S. Song, B.S. Khirabadi, F.G. Lightfoot, K.G.M. Brockbank, M.J. Taylor, Vitreous cryopreservation maintains the function of vascular grafts, Nature Biotechnology 18 (2000) 296-299.
14. Y.C. Song, Y.H. An, Q.K. Kang, C. Li, J.M. Boggs, Z. Chen, M.J. Taylor, Vitreous preservation of articular cartilage grafts, J. Invest. Surg. 17 (2004) 65-70.
15. W.J. Armitage, S.C. Hall, C. Routledge, Recovery of endothelial function after vitrification of cornea at -110?C, Invest. Opthalmol. Vis. Sci. 43 (2002) 2160-2164.
16. F. Migishima, R. Suzuki-Migishima, S.Y. Song, T. Kuramochi, S. Azuma, M. Nishijima, M. Yokoyama, Successful cryopreservation of mouse ovaries by vitrification, Biol. Reprod. 68 (2003) 881-887.
17. M. Salehnia, Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant, Exp. Anim. 51 (2002) 509-512.
18. G.M. Fahy, Vitrification as an approach to cryopreservation: General perspectives, Cryobiology 51 (2005) 348. [Abstract]
19. Y. Pichugin, G.M. Fahy, R. Morin, Cryopreservation of rat hippocampal slices by vitrification, Cryobiology 52 (2006) 228-240.
20. J. Lemler, S.B. Harris, C. Platt, T.M. Huffman, The Arrest of Biological Time as a Bridge to Engineered Negligible Senescence, Ann. N.Y. Acad. Sci. 1019 (2004) 559-563.
21. J.M. Baust, M.J. Vogel, B.R. Van Buskirk, J.G. Baust, A molecular basis of cryopreservation failure and its modulation to improve cell survival, 10 (2001) 561-571.