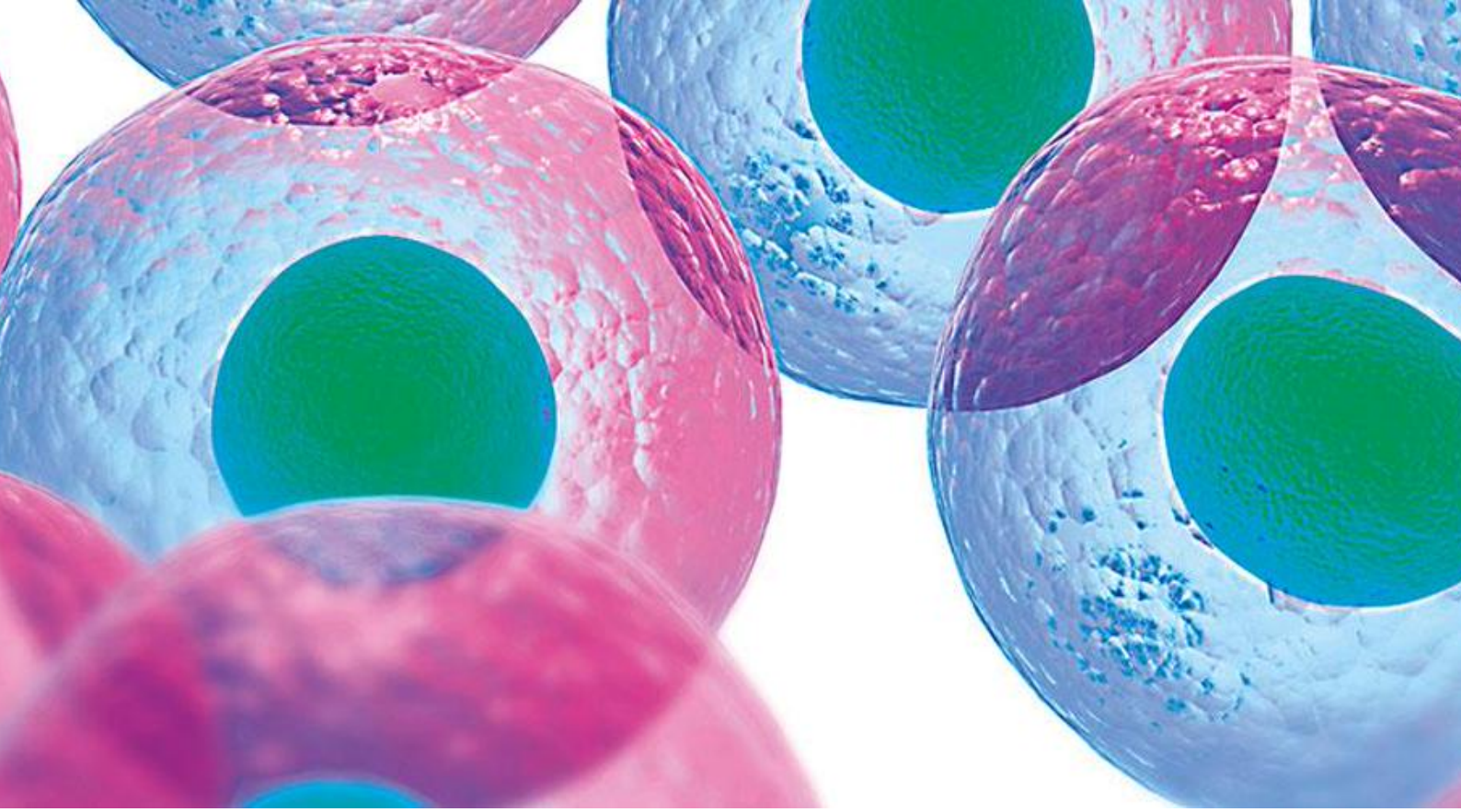

Cryoprotection: ask the experts

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In this 'Ask the Experts' feature, a panel of key thought leaders share their perspectives on current obstacles and future developments facing the cryoprotection field. Discover more about this from our panelists, Oren Ben-Yosef (Sartorius, Beit HaEmek, Israel), Barry Fuller (University College London and Royal Free London NHS Trust, London, UK) and Adam Higgins (Oregon State University, OR, USA).

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How do cryoprotectants work and why do they play a significant role at deep cryogenic temperatures?

Oren Ben-Yosef
Biological Industries

Cryoprotectants have two main roles: the first is to prevent the formation of ice crystals (either intracellular or extracellular) and therefore prevent rupture of the cells' membrane, either from the inside out or vice versa by replacing the water molecules in the cytoplasm with the cryoprotectant. The second function of the cryoprotectants is to keep the osmolarity of the cells at normal levels. During freezing the external ice formation causes the cells to dehydrate and thus undergo apoptosis, and one way of preventing this is by using cryoprotectants with a high molecular weight, thus preventing the 'leakage' of water out of the cells.

Barry Fuller
University College London and Royal Free London NHS Trust

Cryoprotectants are multifactorial in their range of activities, but one overriding property is the ability to modulate the water–ice transition as temperatures are lowered during cryopreservation. Ice crystallizes from pure water, leaving the cells and the original solutes present in the medium (such as salts, amino acids, sugars in a typical tissue culture medium) in a smaller liquid volume.

As ice continues to grow during cooling, the residual liquid becomes increasingly concentrated, which has significant impact on cell molecular organization and leads to cell death. By modifying how liquid water interacts with the growing ice crystal mass, and the final ice crystal fraction produced during deep cooling, cryoprotectants can mitigate against the lethal cell injuries.

Another major effect of cryoprotectants is to reduce the possibility of ice crystals growing within intracellular compartments during cooling, which also helps in avoiding cell death. All of these effects are mostly observed during cooling down through intermediate sub-zero temperatures (around -40°C). If the cells can be cooled to deep cryogenic temperatures (below -100°C) without injury, the whole sample matrix (ice, cells, cryoprotectants and other solutes) undergoes a glassy transition, whereby all molecular and biophysical interactions are halted and the cells can be stored for very extended periods of time (for more than 10 years) if required. An alternative cryopreservation strategy (called vitrification) attempts to achieve this glassy transition without formation of any ice, but this requires extremely high concentrations of cryoprotectants (in excess of about 4 mol l-1), which considerably raises the possibility of toxic effects from the agents themselves (see discussion below).

Adam Higgins
Oregon State University

To understand how cryoprotectants work it is helpful to understand the mechanisms of damage during cryopreservation.

The most common cryopreservation approach is slow freezing, in which the sample is exposed to extracellular ice during cooling to cryogenic temperatures. Ice can cause damage directly by interacting with cellular structures, or indirectly by removing water from the unfrozen solution and thus increasing the concentration of solutes. This latter mechanism of damage is known as solution effects. Cryoprotectants can prevent damage by modifying ice structure, inhibiting ice formation (e.g., by promoting formation of a glass) or by mitigating solution effects.

What factors need to be considered when selecting or developing a cryoprotective agent?

Oren Ben-Yosef

These are two separate questions with two distinct entities that have different considerations. Selecting a cryoprotective agent or solution is done by the end-user, who can be a researcher, scientist in R&D departments or process engineer in the pharmaceutical industry to name a few. Their considerations can range from price, to viability post-thaw, to shelf life and many other parameters. A chief parameter, for instance, would be whether the cryopreservation solution has been tried on their specific cells.

Developing a cryoprotective agent or solution entails, among other factors, the actual cryoprotectant (e.g., DMSO) and its concentration, as this will have an impact on the target end-user and market; the QC process to validate the cryopreservation solution, as this will impact what cells are intended to be frozen using this solution, in what concentration, including the expected viability, etc. These factors will also include the end-user's considerations, as this is part of product development ("Voice of the Customer").

Barry Fuller

There is no single chemical classification that identifies an agent as a successful cryoprotectant. As discussed above, the ability to interact with water molecules in a reversible way and inhibit to some extent them joining ice crystals, is important. The ability to be cooled to deep temperatures in aqueous solutions without themselves crystallizing out is also important. There are two broad categories of cryoprotectants, those that can modulate water–ice interactions AND additionally penetrate across cell membranes to stabilize processes inside the cell (often termed intracellular cryoprotectants); and secondly, other cryoprotectants that remain only in the extracellular spaces. There is a general consensus that, however these two classes of agents may be be used in specific circumstances, there is always a need for some fraction of the mixture to act as an intracellular cryoprotectant.

Given that relatively high concentrations of cryoprotectants are generally required (about 1 mol l-1), and normal cellular structural and molecular processes depend on water, there is a risk that cryoprotectants can themselves injure cells and become toxic. Such negative impacts need to be investigated when developing any new cryoprotectants.

Adam Higgins
Design of new cryoprotectants should be guided by the mechanism of damage that is being targeted. For example, there have been some recent reports of new molecules that inhibit ice recrystallization. These molecules have been shown to be effective at preventing ice damage, even at low concentrations. If the goal is to prevent damage due to solution effects, then it is likely that the cryoprotectant will need to be used at a relatively high concentration. DMSO, for example, is usually most effective at a concentration of about 10%. To design an alternative to DMSO, desirable chemical properties include low toxicity, low molecular weight, high solubility in water and high cell membrane permeability.

How might the percentage of DMSO in cryopreservation media influence toxicity/effectiveness?

Oren Ben-Yosef
DMSO is the cryoprotectant of choice for most of the commercially available (and homebrew) cryopreservation solutions (like NutriFreez D10, which contains 10% DMSO). It is small enough to enter the cells and replace the water molecules in the cytoplasm, thus preventing the formation of ice crystals and maintaining homeostasis. Due to its permeability, it can be toxic to cells at higher concentrations, or at longer exposure times. The usual concentration of DMSO is 10% or lower, as higher percentages tend to vastly affect the viability of the cells during freezing and/or thawing. Therefore, decreasing the percentage is beneficial to the cells but other cryoprotectants (either external or internal) will have to replace the decreased percentage of DMSO, and some of those might be just as harmful for the cells (like ethylene glycol, for instance). Biological Industries, now a part of Sartorius' Advanced Therapies Division, is developing a 5% DMSO cryopreservation solution that has no other harmful cryoprotectants.

Barry Fuller
The concentration of any cryoprotectant (such as DMSO) has the potential to produce negative impacts on normal cell functions by modifying essential water relationships, whether they be structural or metabolic. Thus, it is essential to establish the minimum concentration required for consistent successful cryopreservation. It is also important to establish the methods for loading and unloading cryoprotectants into the cell mixtures with the avoidance of osmotic injuries before and after cryopreservation.

The combined knowledge surrounding the shortest efficacious exposure times, and the lowest concentration to achieve success, will inform the optimization of cryoprotectant protocols. Some of these factors can be developed into predictive modeling, but currently it is not possible to model all of the different impacts into one overarching model.

Adam Higgins

Typically, DMSO at concentrations ranging from 5–15% have been shown to be effective for cryopreservation. Completely removing DMSO from the cryopreservation medium generally results in significant cell damage. Thus, a minimal amount of cryoprotectant is needed. The ideal cryopreservation medium contains the minimal amount of cryoprotectant necessary to mitigate solution effects, but no more than this minimum amount to reduce the potential for toxicity.

In your opinion, how could issues surrounding DMSO toxicity be mitigated?

Oren Ben-Yosef

As I mentioned before, there are alternatives to DMSO that are being considered by the major players in the cryopreservation market, some as 'harmful' to the cells, some more so and some less.

Another good way to mitigate the cytotoxicity of DMSO is by applying good cell culture practices (GCCP) – remembering the adage: "slow freeze, quick thaw" will help the recovery of the cells, for instance. This means that you must have some sort of control over the freeze rate (usually 1°C/min) by using controlled rate containers (e.g., Mr Frosty) or automated freezers/thawers (e.g., Sartorius' Celsius® S3 Benchtop Freezing Platform), and also that you remember to thaw the cells as quickly as possible in order to shorten their exposure time to the DMSO in the cryopreservation medium when in liquid form.

Barry Fuller

Given that there are several potential negative impacts of exposure to DMSO (or any other cryoprotectant), as discussed above, it is important to select the lowest successful concentration for cryopreservation, and the optimized steps for loading and unloading before and after cryopreservation. Reducing the temperature of exposure can also reduce the kinetics of interaction of DMSO with cellular molecular structures, but is counter-balanced by slowing the intracellular permeation of the cryoprotectant (so effectively prolonging the necessary exposure time). These considerations may differ between different cell types and increased complexities of the target cell product – for example, if complex tissues or organoids are required to be cryopreserved, rather than single cells in suspension. Lastly, DMSO concentrations (and thus potential toxic effects), can be reduced by adding a secondary cryoprotectant (such as an extracellular sugar, often oligosaccharides) that can help to modify water–ice transitions.

Adam Higgins
One option for mitigating DMSO toxicity is to use a different cryoprotectant. Besides DMSO, various other similar cryoprotectants are routinely used in cryopreservation, including glycerol, ethylene glycol and propylene glycol. Glycerol is already present in our bodies at low concentrations and has been shown to be less toxic than DMSO in some cases. However, the drawback of glycerol is that it permeates cell membranes relatively slowly, which makes cells more susceptible to osmotic damage.

Another option to mitigate DMSO toxicity is to remove DMSO from the product post-thaw. Various technologies have been developed for this purpose, including microfluidics-based washing devices and centrifugal cell washers.

What advancements have been made into serum-free alternatives for cryoprotective agents?

Oren Ben-Yosef
Serum is not a cryoprotectant! While it does have high concentrations of proteins and salts that help keep the osmolarity and prevent the formation of extracellular ice, it is much too variable lot-to-lot, and much too undefined, to be considered a cryoprotective agent. Therefore, most commercial companies that deal with cryopreservation have some sort of serum-free, xeno-free or even animal component-free cryopreservation solution. The serum is usually replaced with some high molecular weight molecule, such as methylcellulose in NutriFreez. This makes the solution more defined, removes the lot-to-lot variation and also helps the cells adjust better to the cryopreservation procedure.

Barry Fuller
Serum proteins may have multiple positive impacts on cell growth and function, but when thinking about cryopreservation, one main impact is for the proteins also to normalize cell water relationships. Thus, an impact as an extracellular cryoprotectant has often been considered beneficial. Other macromolecular polymers (such as polyethylene glycol, dextran, purified albumin) can also behave as extracellular cryoprotectants. These have been used in a variety of cryoprotectant formulations. However, since serum proteins are of themselves a complex mixture, it is currently unclear if we have yet achieved a comprehensive replacement strategy suitable for all cell types.

What are the challenges associated with implementing serum-free production strategies and how might they be addressed?

Oren Ben-Yosef
Since serum is a 'black box', we can't be sure what it contains. Even with a lot of the parameters and components being tested as part of the Certificate of Analysis (CoA), there is much more that is unknown and untested. Therefore, when moving to serum-free conditions, one has to take into account what are the most common components that need to be replaced and what can be added to the media to get it to support cell expansion and proliferation. Most common components include insulin, transferrin and sodium selenite, commonly know as ITS. But specific growth factors are also needed for certain types of cells, such as bFGF and TGFβ for stem cells. When producing serum-free cryopreservation solutions, as I stated before, serum is not a cryoprotectant and therefore replacements for it are in order, which include DMSO, methylcellulose and other cryoprotectants.

Barry Fuller
Whilst we are gaining a better understanding of the roles of non-serum macromolecules as cryoprotectants in the biophysical aspects of cryopreservation, the more ephemeral impacts of growth factors that might be associated with serum proteins have yet to be fully understood specifically in terms cryopreservation itself (i.e., can specific signaling mechanisms be enhanced or lost if serum proteins are in the mixture). For some cell systems, successful cryopreservation can be achieved without serum in the mix, but in others, perhaps better post-thaw functions may be achieved if specific cell signaling mechanisms can be enhanced; our current knowledge is incomplete.

What is clear is that there are subtle impacts of cryopreservation that can be detected out to 1 or 2 days of recovery – the so-called cryopreservation induced, delayed onset cell death. It is hoped that eventually it will be possible to help cell recoveries by providing specific signaling agents during the early post-thaw period (and thus potentially remove the need for serum proteins as cryoprotectants).

How could switching to a salt-based solution rather than media-based help with producing a more defined cryopreservation solution?

Oren Ben-Yosef
Most 'homebrew' cryopreservation solutions are made by using the complete growth medium of the cells (i.e., DMEM, RPMI, DMEM/F12 etc., 10% FBS and antibiotics) and adding DMSO to it, usually 10%. This works, for the most part, and has worked for many years. But this is not optimal, not for academia and definitely not for clinical applications and cell therapies. When going towards clinical application, the more defined reagents used the better. Basal media, while being very simple in composition, still contain vitamins, amino acids and other components not relevant to cryopreservation.

Therefore, moving towards more defined, clinically relevant solutions, ones that are based on salt solutions, like saline, is a paramount consideration both by the end-users and the manufacturers.

Barry Fuller

A range of cryoprotectant-carrier solutions have been investigated in recent years. For example, use of organ preservation solutions in transplant services. The University of Wisconsin (WI, USA) organ preservation solution is one such solution that has been used in cryopreservation, but there are others. One advantage for specific cryopreservation applications is that the solution is widely used and deemed safe in clinical services across the globe. Such solutions are also commercially available and have regulatory approval and consistent production for clinical use in many countries.

Lastly, how do you see the use of cryoprotectants evolving over the next 10 years?

Oren Ben-Yosef

The next logical step in the use of cryoprotectants is moving to DMSO-free cryopreservation solutions. This means that the DMSO must be replaced with some other cryoprotectant.

While there are manufacturers that are ostentatiously currently offering such solutions, those solutions contain other cryoprotectants that, while are not DMSO, are equally as harmful to the cells, like ethylene glycol. Therefore, a solution must be found for 'true' DMSO-free cryopreservation solutions, and our R&D team is working on such as we speak.

Another development would probably be the emergence of automated freezing/thawing apparatus and having them being more readily available not just to the larger companies and the industry, but also to research labs and academic institutes. That would make everything more regulated and more reproducible, and less dependent on specific users.

Barry Fuller

As cryopreservation sciences progress and become an integral part of the delivery pathways for regenerative medicine, there will be increasing focus on how cryoprotectants modulate the cell molecular changes that are a consequence of deep cryogenic storage. Powerful physical tools such as neutron diffraction, cryo-electron microscopy and modulated differential scanning calorimetry are already being used in these ways. For example, such studies are leading to the development of novel chemical classes that can impact on the propensity for water to nucleate ice at low temperatures, and which will contribute to a more comprehensive cryoprotectant strategy. The 'omics' sciences will give us a deeper understanding of which cell pathways have been impacted by

cryopreservation, and signpost development of enhanced cryoprotectant agents that may be able to control cell death pathways and on the other hand, promote repair mechanisms post-thaw. It is now some 60 years since there was a concerted effort to identify agents that might offer cryoprotection, and I believe that we are now moving towards a 'second-era' understanding of how to reap most benefits from selection of cryoprotectants that can be better targeted towards specific requirements for long-term storage of cells and tissues.

Adam Higgins

There is tremendous potential for expanding the repertoire of cryoprotectants over the next decade, which may make it possible to cryopreserve complex samples like organs, or even whole organisms. Recent work with ice recrystallization inhibitors is very promising. Some new molecules have even been shown to be cell membrane permeable and capable of preventing ice recrystallization intracellularly. Recent work also demonstrates the potential for leveraging advances in molecular biology to discover genetic mutations that confer resistance to cryoprotectant toxicity. This work could lead to new drug-like cryoprotectants that target particular genes. There is also great potential to expand the number of conventional cryoprotectants beyond the small number that are currently in use (e.g., DMSO, glycerol) and to identify less toxic mixtures of cryoprotectants for cryopreservation.

Meet the experts

Oren Ben-Yosef
Technical Support, Sartorius (Beit HaEmek, Israel)
Oren Ben-Yosef has a BSc in Biotechnology Engineering and a PhD in Medical Sciences from the Technion Israel Institute of Technology (Haifa, Israel). He conducted his research in the laboratories of Prof. Ido Perlman and Prof. Joseph Itskovitz-Eldor, focusing on embryonic stem cells and induced pluripotent stem cells culturing methods and differentiation. Oren joined Biological Industries, which is now part of the Sartorius group, in 2016 as part of Scientific and Technical Support. He has been Product Manager for Stem Cells since 2019.

Barry Fuller
Professor of Surgical Sciences and Low Temperature Medicine, University College London and Royal Free London NHS Trust (London, UK)
Barry Fuller is a Professor of Surgical Sciences and Low Temperature Medicine within the Division of Surgery and Interventional Sciences at University College London and Royal Free London NHS Trust. His career-time focus has been applications of low-temperature technologies in biology and medicine, with the major efforts directed to preservation of cells, tissues and organs for clinical therapies. These studies have encompassed both non-freezing cold preservation and cryopreservation at deep cryogenic temperatures. He worked for many years in the clinical donor organ preservation service within the transplant services at the Royal Free (London, UK), where chilling with aid of specialized organ preservation solutions allowed organ sharing across the UK transplant center networks.

Adam Higgins
Associate Professor, Oregon State University (OR, USA)
Adam Higgins is an Associate Professor in the School of Chemical, Biological and Environmental Engineering at Oregon State University. He currently serves as President of the Society for Cryobiology (MD, USA). His research focuses on stabilization of biomolecules, cells, tissues and organs using technologies such as cryopreservation, lyophilization and spray drying, with a particular emphasis on mathematical modeling and optimization to improve outcomes and reduce cryoprotectant toxicity.