

Alternatives to cryopreservation for the short and long-term storage of mammalian cells

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Abstract

The ability to preserve mammalian cells has long been a critical part of cell-based research for several reasons. It allows for the transport of cells between laboratories or sites, ensures the availability of consistent starting material for the research through the establishment of cell banks and can uncouple cell-based assays from the culture process. Although cryopreservation, whether conventional slow freezing or vitrification, is widely used as a method for preserving cells long-term, it can result in low cell recovery post-thaw and the cryoprotective agents used in the freezing medium can be cytotoxic. With the emergence of a cell-based therapies industry, where clinical grade cells will need to be stored and transported, there is a growing need to develop scalable GMP-compatible preservation methods that retain not just high cell viability but also clinical efficacy. With these issues in mind, this review will explore two alternatives to cryopreservation: cell desiccation for long-term storage of cells and the short-term storage of cells under hypothermic ($>0^{\circ}\text{C}$) conditions.

Introduction

Cell-based therapies are part of a growing healthcare industry and are estimated to be worth \$5 billion by 2014 (1). Such treatments have typically involved harvesting material such as bone marrow or islet cells from donors and transplantation or transfusion of freshly isolated or expanded cells. Cryopreservation, however, enables 'off the shelf' allogeneic therapies to be generated as the product, i.e. the cells, can be manufactured at a site physically separate from the clinic, stored and shipped as required. It also allows the generation of master and working banks of cells for allogeneic therapies and time for extensive QC and safety testing to be done before a product lot is released. Examples of cryopreserved cell therapies currently in clinical trials or use include CTX0E03 neural stem cells (ReNeuron Ltd, UK; (2)), Prochymal® (Osiris Therapeutics Inc, USA; (3)) and Dermagraft® (Advanced BioHealing, USA; (4)). Cryopreservation also underpins the growing business of cord blood banking. Despite its widespread use, there are several issues associated with the cryopreservation of cells and these will be briefly discussed before two alternatives to cryopreservation, namely cell desiccation and hypothermic storage, are explored.

Issues associated with the cryopreservation of mammalian cells.

Although cryopreservation, whether conventional slow freezing or vitrification, is widely used as a method for preserving cells long-term, it can result in low cell recovery post-thaw. For instance, several studies reported poor survival and recovery rates of between 0 and 30% of human embryonic stem cell colonies or clumps when a slow freezing protocol was used (5-8). Furthermore, although cell survival or viability is clearly an important measure of cryopreservation success, it is also important that cells retain their function long-term post-thaw, particularly when considering clinically relevant cells. For example, protocols have now been developed that enable cryopreservation of human hepatocytes from donor livers for cell transplantation. Although high numbers of viable cells can be recovered post-thaw, large proportions of cells lose their ability to attach to surfaces which is key during engraftment, and their metabolic functions are altered (9), indicating further improvements still need to be made.

Key to the success of any cryopreservation protocol is the use of cryoprotective agents (CPAs). One of the most commonly used CPAs is DMSO, despite the fact that it is reported to be toxic to cells (10) and has been linked to cell differentiation (11). Cells therefore need to be processed rapidly and ideally at low temperatures. Although this may be practical on a small scale, this becomes more difficult when processing cells at an industrial scale, which could take upwards of an hour (12). Furthermore, DMSO has also been reported to cause adverse reactions in patients (13) and so cells may need to be washed extensively prior to infusion or transplantation into patients. This extra processing step can result in significant cell losses (14), thus it is clear that protocols that avoid or minimise the use of DMSO must be considered, especially for preservation of cell-based therapies.

Given the large numbers of cells that may be required for allogeneic cell-based therapies (15) it is also important that cryopreservation protocols are developed that are scalable and GMP compliant. Although conventional slow freezing protocols are adaptable, vitrification is not a truly scalable process (10, 15, 16). Vitrification also poses extra risks as cells in open cryocontainers are exposed directly to liquid nitrogen which can contain contaminants such as microorganisms (17). With all frozen cells, whether conventionally frozen or vitrified, it is also important that they are stored appropriately, with no fluctuations in temperature (10, 18). This means careful environmental control is required, especially during transport. Transport of vitrified cells can, for instance, be very expensive as dry shippers, which hold samples at -150°C without the risks associated with transporting liquid nitrogen, are required to prevent any partial thawing of the cells (10, 18).

Notably, a great deal of research is on-going to improve current cryopreservation protocols and overcome these issues. Some of these innovations have recently been reviewed elsewhere (10, 15, 19) but, for example, using BHK cells, the impact of prolonged exposure (up to 5 hours) to DMSO on cell recovery and therefore what the true processing window for banking large numbers of cells is, was determined (12). The authors found that if using cryobags the banking should be done within 1.5-2 hours at room temperature. Furthermore, Hunt and colleagues found that CD34⁺ cells could be exposed to 25% (w/w) DMSO for up to 1 hour at 2°C with no significant loss in the cells ability to generate colony forming units (20). Although the authors only looked at exposure of freshly isolated cells to DMSO and not the impact

that long-term exposure followed by freezing has on cell recovery, it is nonetheless encouraging to see that process conditions are starting to be defined beyond simply 'work quickly'. The use of closed cryocontainers for vitrification to avoid direct exposure to liquid nitrogen is also being investigated (7, 21), as are 'bulk' vitrification methods (22), although even these are still relatively small scale (15). Thus, although improvements are being made to the cryopreservation methods, it is worth exploring whether a preservation method, such as desiccation or hypothermic storage, which results in products that can be maintained at room temperature or in a refrigerated unit and could avoid the use of DMSO altogether, are viable alternatives.

Alternatives to Cryopreservation:

Dry preservation

One alternative to cryopreservation is to avoid low temperature storage and transport altogether by desiccating cells as dried formulations could be kept at room temperature. If feasible this would be both economically and practically advantageous, especially for long-term storage of cells. Although drying cells generally leads to membrane damage, protein denaturation and subsequent cell death, the inherent capacity of some organisms such as yeast cells and fungal spores to survive almost complete dehydration (23) suggests 'safe' dehydration and rehydration of mammalian cells should be possible. Here efforts to both freeze dry (lyophilise) and simply desiccate cells by, for instance, vacuum desiccation, will be reviewed.

Freeze drying, or lyophilisation, is typically used to preserve bacterial cells and fungi. Briefly, the process comprises three stages: freezing in order to partly crystallise the solvent so that it can be separated from the solutes, sublimation of the resultant ice (primary drying phase) and finally, a secondary drying phase where the majority of the remaining moisture is removed by desorption (23, 24). Just as cryoprotectants are used in cryopreservation, cells can be pre-treated and indeed lyophilised in the presence of lyoprotectants, to minimise cell damage during the process. One compound that has been particularly researched is trehalose, a hydrophilic disaccharide which is known to accumulate in desiccation tolerant organisms (25).

The key challenge in using trehalose as a lyo- or indeed cryo-protectant is that it is membrane impermeable and thus achieving the intracellular concentrations of at least 100-200mM required for protection (26, 27) is non-trivial. Several different approaches have been reported including using fluid phase endocytosis (28), microinjection (29), mutant bacterial toxins as pore forming proteins (26, 27), genetically modifying cells to synthesise trehalose themselves (30) or express a trehalose transporter on their plasma membranes (31), osmotic shock (32, 33), electroporation (34) and a biopolymer (PP-50; (35)). How trehalose is currently understood to exert its protective effects has been thoroughly reviewed in Crowe *et al* (25) and Ohtake and Wang (36) but includes its role as a water replacement inside cells and its effectiveness as a stable vitrification agent.

Efforts to lyophilise mammalian cells have largely focused on trying to generate freeze-dried blood, with some studies showing that erythrocytes and platelets can be successfully lyophilised, stored and rehydrated, although the processes are not yet optimal (28, 37-40). For instance, Goodrich *et al* (37) reported that based on their patented method of erythrocyte lyophilisation, which involves lyophilising cells in the presence of monosaccharides such as glucose, cells maintained their metabolic function following rehydration although they note that it takes several days to reach the 1-2% residual moisture content in the samples that allows for storage at ambient temperatures. By chemically cross-linking cells in a reversible manner, Bakaltcheva and colleagues (39) were also able to successfully lyophilise erythrocytes, with less than 10% haemolysis reported in their optimal conditions. However, a final moisture content of 10-15% meant that cells had to be stored at 4°C, not ambient temperatures. At moisture contents of less than 10%, haemolysis upon rehydration increased significantly and the authors themselves raised concerns over the safety of transplanting these chemically treated cells into patients (39). Platelets have also been successfully lyophilised using trehalose as a lyoprotectant though it has been noted that the density at which cells are lyophilised and the method of how cells are rehydrated have a significant impact on the success rate (28). Nonetheless, results are promising as a recent study using a model of deep arterial injury has even shown that lyophilised human platelets, when reconstituted into platelet free plasma, retained their haemostatic properties (40).

Although the genetic material of mouse embryonic stem cells, spermatozoa and sheep granulosa cells has been preserved following freeze-drying (41-43), it was not until relatively recently that successful lyophilisation, storage for one week, and rehydration of nucleated cells was reported (44). The authors demonstrated viability of mononuclear cells isolated from human umbilical cord blood following rehydration, with similar levels of CD34⁺ haematopoietic stem cells present in the population before and after preservation, which were capable of differentiating into a number of cell types. In another study also focusing on haematopoietic progenitors, cells rehydrated after 4 week of storage at 25°C also retained their ability to generate colony forming units of different lineages. Rehydrated cells produced between 35-80% colony forming units relative to unprocessed controls, depending on the lineage studied (45).

For a number of therapies to be effective, cells may need to be encapsulated in order to, for instance, protect them from the patients' immune system (46). Therefore, the ability to freeze dry microencapsulated cells has also been investigated (47). The viability of human retinal pigment epithelial cells, either dispersed or microencapsulated, was determined before and after the drying step and, although it was clear that drying drastically reduced viability of cells compared to freezing alone, this proof of principal study indicates that encapsulated cells could be freeze-dried.

It is also possible to simply dry cells, under vacuum for instance, without the need for freezing. As with lyophilisation, the use of a protective agent such as trehalose appears to offer protection (32), although cell injury/death can still occur if the drying process is too slow or not uniform enough, thereby exposing cells to severe osmotic imbalances (48, 49). It has been shown that human mesenchymal stem cells (MSCs) exposed to trehalose and glycerol can be successfully dehydrated under vacuum and stored for 1 day (50). Upon rehydration they regained their normal morphology and adhesive capability, were >90% viable, proliferated and maintained expression of key surface markers. However, the authors did admit that there was a large amount of inconsistency from trial to trial and that longer storage times would need to be achieved. As they did not measure the levels of intracellular trehalose achieved it is possible that variation in this between runs or simply insufficient trehalose loading overall affected the success rate of desiccation. After expressing a trehalose transporter in CHO-cells, one group also reported that they could be successfully

desiccated after loading with trehalose (31). Membrane integrity was retained in approximately 80% of cells when moisture content was reduced to as little as 2.6 g water/g dry weight. However, upon rehydration these cells exhibited a vastly reduced proliferative capacity compared to control cells. Given that only ~23.5 mM intracellular trehalose concentrations were achieved, well below the 100 mM minimum mentioned earlier, it may be that with enhanced trehalose uptake the desiccation tolerance of CHO cells could also be improved.

Although these reports are promising, indicating that desiccation of cells for long-term storage may be possible in the future, there are several caveats. Clearly, as already mentioned, storage times must be increased. Secondly, if cells are to be used in cell therapy, it is important that cells are not simply viable immediate post-rehydration but retain normal function and clinical efficacy. For example, one study reported that human bone marrow-derived MSCs could be freeze-dried (51) but, whilst initial recovery following rehydration was high (~70%), cells were unable to adhere to tissue culture plastic as normal and died within 1 week. Similarly, Li and colleagues (52) successfully lyophilised Hep2G cells loaded with trehalose and modified to express two late embryogenesis abundant proteins which are associated with desiccation tolerance in anhydrobiotic animals and bacteria. A very high level of membrane integrity was retained up on rehydration (98%), but cell growth was decreased compared to non-dried controls. These studies highlight the need to look at the longer-term effects of these processes, not just immediate post-rehydration viability as the longer-term health of the cells may be compromised.

Based on their findings in human foreskin fibroblasts, Puhlev *et al* (32) suggested that free-radical mediated damage may occur within desiccated cells. In order to achieve successful desiccation of nucleated cells we may therefore need to develop more complex protocols in order to overcome or prevent some of the stress that cells undergo during freezing and/or desiccation. For example, Chakraborty and colleagues have looked at ways to improve the drying process in order to reduce the osmotic stress that cells undergo. They developed a spin-drying technique (49) which enabled a much lower water content to be achieved (0.16 g water/g dry weight) than in other studies, whilst retaining more than 95% membrane integrity of CHO cells. However, the authors do admit that this is preliminary data and the longer-term impact of such extreme desiccation on the CHO cells was not investigated. The impact of including additional lyoprotectants has also been explored. For instance,

one study transfected HEK293 cells with a stress protein, p26 from *Artemia*, and found that it has a synergistic effect with trehalose in improving cell viability following air-drying and rehydration. An approximately 15% increase in membrane integrity was seen by the addition of the p26 as well as an increase in cell growth when cells had been dehydrated to 0.2 g water/g dry weight (53).

Although these studies are interesting and show that improvements to protocols can be made, when working with clinically relevant cell types it is critical to remember that the cell is the product and will end up in a patient. It will therefore be important to ensure that any additives used, or even the processes by which trehalose is loaded into cells, are accepted by the regulator. It is important to note that lyophilisation has been used to preserve bacterial cells (54) and its use in the pharmaceutical industry means that there is a precedent for lyophilisation at a large scale, making it an attractive option which should continue to be investigated.

Hypothermic Preservation

Another potential alternative to cryopreservation for mammalian cells is hypothermic preservation where cells are stored at low but not freezing temperatures, effectively pausing the cells by slowing down metabolism, but without the dangers of ice formation. At mild hypothermia (25-35°C) cells can still proliferate and grow and in fact, recombinant protein expression may be improved by culturing cells at reduced temperatures (55). More severe hypothermia and refrigerated storage (0-10°C) is needed to arrest growth and pause the cells but this would nonetheless be preferable to storing cells in liquid nitrogen and the use of dry shippers. This would be a short-term preservation method and indeed, it is already used in the pharmaceutical industry as it can uncouple cell culture from cell-based assays (56). It allows researchers to store cells for several days if a piece of equipment is double-booked or breaks down, rather having to dispose of plates of cells, saving time and money. Just like cryopreservation, hypothermic preservation also uncouples production or harvest of cells for cell-based therapies from their clinical use. Although shelf life is more limited than with frozen products, this allows for an 'off the shelf' business model to exist, QC testing to be completed before cells are released and avoids the need for continuous culture.

Organs and blood fractions have long been stored at hypothermic temperatures prior to transplantation or transfusion (57, 58), however, cold injury can occur which can be amplified when cells are rewarmed. The changes that cells undergo during cold exposure include: decreased membrane fluidity, changes in pH, osmotic imbalances, activation of the unfolded protein response, oxidative stress (59, 59, 60) and often lead to the initiation of apoptosis. Therefore, several cold storage media have been designed to improve organ, tissue and cell preservation at temperatures in the range of 2-10°C. These include University of Wisconsin Solution (UWS, also known as Viaspan), EuroCollins, Celsior, Custodiol (also known as Bretschneider's HTK solution) and the HypoThermosol range (60-62). Importantly, HypoThermosol-FRS (BioLife Solutions, USA) is a protein and serum free solution which is manufactured to cGMP standards so that it is suitable for use in cell therapy products. As the formation of reactive oxygen species is often implicated in cell injury, these solutions typically contain antioxidants such as glutathione (60, 63) as well as mimicking the ionic composition of the cells under cold temperatures, providing a pH buffering system and some nutrients. As an example the base formulation of HypoThermosol is given in Table 1 but it is notable that solutions containing iron chelators (60), apoptosis inhibitors (64) and even antifreeze proteins (65) have been tested.

Hypothermic preservation of a range of cell types has been investigated, including hepatocytes (60, 66), MSCs (67), red blood cells (58), neurons (68), renal cells (69) and endothelial cells (59, 62). Despite the use of specially formulated preservation media, cell recovery from hypothermic storage can still be variable and is very dependent on the medium composition. For example, survival of human hepatocytes seeded in monolayer culture and stored at 4°C for up to 1 week in UWS was less than 20% (60). An optimised solution based on Custodiol and containing iron chelators, however, improved cell survival drastically such that cell death was less than 20% (60). Similarly, in a study using several cells types, Mathew and colleagues found that each was best preserved in a slightly different HypoThermosol variant (62). For instance, HypoThermosol supplemented with Trolox and RGD peptide best supported the survival of skeletal muscle cells stored in suspension at 4°C for 7 days whereas hepatocytes were best preserved in a formulation supplemented with a caspase inhibitor, FK041.

Hunt and colleagues (70) also demonstrated that it is possible to store cells in their normal culture medium. They reported that recombinant CHO and HEK293 cells (both adherent and suspension) could be paused for up to 3 weeks over a wide range of temperatures, from 4 to 24°C. Cells retained their capacity for growth and protein production once rewarmed to 37°C. Another study has shown that human embryonic stem cells in culture medium can also tolerate temperatures of 4 or 25°C for up to 48 hours with approximately 70% survival and retention of a normal karyotype and expression of pluripotency markers (71). This does not seem to be universally applicable though as, in contrast, when human bone marrow derived MSCs were stored at 4°C in their growth medium they failed to survive, although they could be successfully stored for up to 4 days in HypoThermosol-FRS (67). Similar results were also obtained by Mathew *et al* when they investigated the preservation of several different cell types (62).

It is important to note that, just as in the desiccation work, some studies have reported that although cell viability following hypothermic storage can be high, cell function can be negatively affected. For instance, storing isolated human hepatocytes in HypoThermosol-FRS at 4°C for up to 72 hours resulted in a cell viability of approximately 70% which was a drastic improvement over cells stored in UWS or the base HypoThermosol medium. However, despite cell viability being high, cell metabolic function, was typically half that of freshly isolated cells (63). Another study which investigated whether bovine limbal epithelial cells could be stored at 4°C in calcium alginate hydrogels found that despite about 50% of cells extracted from the gels post-storage being viable, the colonies formed from these cells were noticeably smaller than those formed from cells that had not been stored (72). These studies highlight that just as with other methods of preservation, it is important to not just focus on cell viability upon rewarming but also cell function and growth.

It is notable that many studies, including some of those referred to here, utilise adherent cells which have been seeded in plates, allowed to reach a certain degree of confluency and the growth medium is then replaced with hypothermic preservation solution before the plate is moved into cold storage. Although this is suitable for cells that will be used in plate-based cell assays such as those commonly used in pharmaceutical research, it is not suitable for storage of cell-based therapies. For example, a clinical dose of MSCs could exceed 2×10^8 cells (73) and in our hands a

confluent T75 contains approximately 3×10^6 MSCs [74] so a minimum of around 29 T175 flasks would be required for a single dose. Aside from the logistics of storing or transporting such large numbers of tissue culture flasks containing the liquid hypothermic preservation medium, it is also easier for the clinicians to receive vials or bags of cells that are ready for transplantation or transfusion, without needing to harvest cells from flasks. It is therefore important that anyone investigating the potential to store cells for cell-based therapies at 4°C does so under appropriate conditions.

Overall, hypothermic preservation does overcome some of the issues with cryopreservation. It avoids the use of DMSO, is amenable to scale up in that cells could be stored in vials or cryobags and GMP compliant preservation media is already available (Table 2). However, results so far have indicated that with some tweaking of the preservation solution, most cells are amenable to hypothermic storage. It will clearly always be a short-term solution compared to cryopreservation or indeed desiccation but, as is already apparent in organ transplantation and the blood banking industry, it has a role in getting cells from the site of harvest or production to the clinic. It remains to be seen how widely applicable hypothermic storage of cells is when the long-term effects on cell function and the impact of preservation in suspension are explored in more detail.

Conclusions and future perspectives

Although cryopreservation is by far the most common method of preserving mammalian cells it is not without its disadvantages. Here, two alternatives, summarised in Table 2, have been presented which could be used for either short or long-term preservation of cells. As hypothermic storage has been used in organ transplantation for so long, this route may be more acceptable to the cell biology community and regulators but desiccation should continue to be explored as an alternative to cryopreservation. As these methods evolve it will also be interesting to see whether, in order to extend the shelf life of products, these preservation methods are combined in the future. For instance, one can envisage a scenario where cells are frozen in the first instance for long-term storage, then thawed, washed and resuspended in hypothermic storage medium for transport to the clinic as and when needed. How cells will respond to these multiple stresses remains to be seen.

Table 1: Formulation of the base HypoThermosol medium. It has an osmolality of 360 mOsm/kg. A vitamin E analogue, Trolox, is added to the base to generate HypoThermosol-FRS (Mathew 2004).

Component	Concentration
Sodium	100 mM
Potassium	42.5 mM
Magnesium	5 mM
Calcium	0.05 mM
Chlorine	17.1 mM
H ₂ PO ₄ ⁻	10 mM
HCO ₃ ⁻	5 mM
HEPES	25 mM
Lactobionate	100 mM
Glucose	5 mM
Mannitol	20 mM
Sucrose	20 mM
Dextran-40	6%
Adenosine	2 mM
Glutathione	3 mM

Table 2: Summary of whether desiccation or hypothermic storage overcome any of the issues surrounding cryopreservation of mammalian cells. TBC; to be confirmed

	Hypothermic Storage	Desiccation
Maintains cell characteristics long-term	TBC	TBC
Widely applicable	Already used for organs and some cellular products	?
GMP compliant	amenable	amenable
Scalable	amenable	amenable
Avoids/limits use of DMSO	✓	✓
End-product is easily stored and/or transported	✓	✓

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