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LIQUIDUS TRACKING: CONTROLLED RATE VITRIFICATION FOR THE CRYOPRESERVATION OF LARGER VOLUMES AND TISSUES

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Abstract

BACKGROUND: Vitrification of cells or tissue at controlled cooling rates suitable for larger volumes, and with reduced cryoprotectant toxicity. **OBJECTIVE:** To set out the current understanding of the LiquidusTracking (LT) vitrification technique, and to discuss the challenges and benefits of translating the method into laboratory protocols more generally applicable to meet requirements of large volume and 3-D cryo-banking in the era of regenerative medicine. **METHODS:** By adding small amounts of cryoprotectants at each step and subsequently cooling the sample just above its freezing point before further increasing CPA concentration, cryoprotectant toxicity is minimized. **RESULT:** CPA toxicity can be reduced by lowering the temperature. Different manual approaches to LT were evaluated and further improved. **CONCLUSIONS:** Manual liquidus tracking is complicated and exhibits potential high variability. Nevertheless, this approach offers the possibility of testing several conditions simultaneously and could be used to pre-test conditions prior to automatic LT development.

Keywords: Vitrification, high cryoprotectant concentrations, Liquidus Tracking, liquidus curve

INTRODUCTION

Since the start of applied cryobiology by the early pioneers (9, 10, 15), a wide range of different cell types have been successfully cyropreserved. This has become an important, routine technology which facilitates exploitation of cell biology for biotechnology, medicine or fundamental research (18). These protocols have been based largely on slow cooling protocols after exposure of the cells to moderate concentrations of cryoprotectants (CPA), with cooling profiles typically in the range of -1°C to -5°C/minute in volumes of 1-2 ml. However, successful cryo-banking of larger structures, such as tissues or organs, remains elusive, with only rare claims of success (5), which have not translated into robust repeatable systems. Even dispersed, cellular systems, in volumes above 10 ml, have required sample size manipulation (e.g. into a thin film volume format (11, 17)). The problems are complex but can be divided into two main areas. The first is the effect of the location and compartmentalization of extracellular ice during slow cooling of 3-dimensional structures. It has been long recognised that ice formation driven by slow cooling tends to proceed in non-uniform ways in larger structures, with surface layers experiencing ice nucleation first (16). The ice tends to accumulate in whatever 'non-structured' liquid spaces are present within the tissue (such as within small capillary blood vessels inside an organ) (8), which act as growing ice foci physically destroying the internal structure of the tissue. The release of latent heat of ice formation deeper inside the structure, causes uncontrolled deviations of the cooling profile resulting potential cryopreservation injury in selected areas. The second major challenge is that, unlike small volume cryopreservation, rewarming

profiles of larger 3-dimensional structures can only proceed at relatively slow rates because of the low heat transfer properties. This compounds the problems of ice crystal re-organisation and growth in the extracellular ice loci within the structure, and associated salt-induced hyper-osmotic injury to the cells as they transition across the higher subzero temperature range.

The technique of vitrification is recognised as a successful way to avoid injury from ice formation (15), and has grown in popularity over the past 10 years in some areas of cryo-banking such as cryo-storage of embryos or oocytes (1, 2). By employing high CPA concentrations (40% – 60% (w/v), successful vitrification protocols can be widely applied (4, 7). However, there are specific requirements for sample volumes (usually less than 200 µl) and high cooling rates which must be imposed, because even at these elevated CPA concentrations, the 'glassy state' is achieved by non-equilibrium glass transitions (2). Ultra-rapid sample manipulation (of a few tens of seconds) and cooling (in excess of -500°C/min) are required to avoid toxic effects from the high CPA concentrations, and kinetically favour the glass transition; cooling at rates achievable with larger 3-D structures (typically <-10°C/min) invariably result in ice formation.

A different approach was suggested some 40 years ago by Farrant (6), based on incrementally increasing the CPA concentration in samples as cooling proceeded, to achieve high enough CPA concentration to avoid ice nucleation, with the required concentration at each temperature being defined by the liquidus curve of the mixture (Figure 1). Farrant's approach to prevent ice formation was principally to avoid solution effects and to maintain a constant electrolyte concentration during the cooling process. Later the benefits of avoiding ice per se became more evident. The technique has been revived and more clearly defined in recent years by Pegg's group, and has become known as Liquidus Tracking (LT) (14, 20). The main advantage of this method is the diminution of CPA toxicity. The liquidus curve (Figure 1) defines the equilibrium melting point temperature for a given CPA mixture (i.e. the highest sub-zero temperature at which ice crystals and liquid can co-exist – and knowing this fact it allows prediction of the lowest subzero temperature which can be reached for that given CPA mix without ice nucleating).

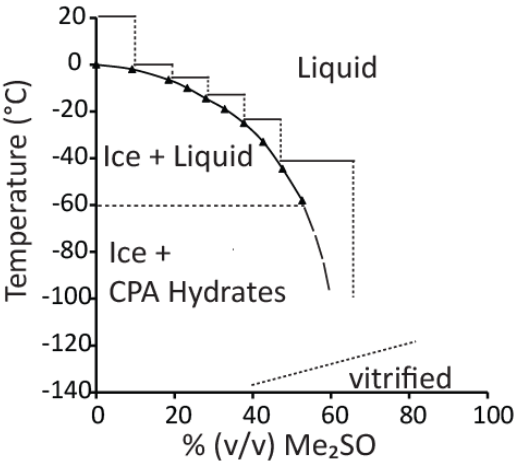

Figure 1. A schematic showing the T_m equilibrium melting curve (liquidus) for Me_2SO (modified from Elford 1970 and Farrant 1965). Arrows indicate a scheme for incremental addition of CPA to obtain the lowest toxicity for a given CPA concentration. This is achieved by decreasing the sample temperature to just above the freezing point (dashed vertical line above the liquidus curve) before adding more CPA (solid horizontal line) to prevent freezing at lower temperatures.

Cryoprotectants are less toxic at lower temperatures (13, 21) and lower concentration. By adding smaller amounts of CPA at each step and subsequently cooling the sample to just above its freezing point, toxicity is minimized when attempting to achieve the very high CPA concentrations required for vitrification. The more increments used, the closer the sample can be kept to the liquidus curve of its carrier CPA solution. Due to reduced CPA toxicity, and avoidance of ice nucleation, samples can be vitrified without the necessity of fast cooling rates, preferable for cooling large volumes, or when longer exposure times are required to allow for sufficient CPA penetration. An example of this strategy is depicted in Figure 1. Of equal importance for these larger volumes is the warming process, which must be reversed in a controlled (stepwise) fashion to dilute the high CPA concentration whilst avoiding any propensity for ice to nucleate.

This review sets out the current understanding of LT, and discusses the practical challenges and benefits of translating the method into laboratory protocols which can be more generally applied to meet requirements of cryo-banking for large volume or 3-D structures in the era of regenerative medicine.

The different technical approaches have been developed using alginate-encapsulated liver cells (AELC) as an illustrative model, and our

group has wide experience in studying these (11, 12). AELC provide a scalable model of complex 3-D cell clusters similar to small tissues, but which can be manipulated in volumes between 1 ml and 1 litre, suitable for investigating LT. The chosen CPA was Me_2SO, which has been the CPA of choice for other recent LT studies (14, 20). The aim was to use AELC as a bio-object to demonstrate the essential technical steps within LT, which might then be tailored for laboratory protocols, rather than provide an in-depth assessment of cryo-banking AELC by this approach.

APPROACHES TO LT OVER THE PAST 30 YEARS

Elford and Walter (20) used the LT approach to study the role of anionic composition and pH of the carrier solution needed to deliver the increasing concentrations of CPA. Isolated strips of taenia coli muscle were progressively cooled to -79°C with a final concentration of 60% (w/v) Me_2SO to prevent freezing. Muscles showed slow recovery and were severely damaged both functionally and structurally when the incubation media had a similar composition to that of Krebs solution, but showed better recovery after rewarming when potassium-rich media containing Na^+, K^+, and Cl^- were used. The degree of recovery was dependent on the size of the anion, showing better contractility of the muscles for glycerophosphate, TES or PIPES (*N*-tris-(hydroxymethyl)-methly-2 amino- or piperazine-*NN'*-bis-2-ethanesulfonates) than for sulfate or ethylsulfate derived anions. When using potassium-rich PIPES media, recovery was lower when the pH was reduced.

The technique lay dormant for many years until it was revived by David Pegg and colleagues (14,20). Pegg used the same protocol as Elford and Walter (but with different incubation times) for LT vitrification of articular cartilage and found that damage was predominantly associated with the formation of ice during standard cryopreservation, which supported the use of a vitrification protocol, but CPA toxicity and the need for rapid warming resulted in inadequate recovery. By using the LT (equilibrium vitrification) approach, cartilage was successfully recovered with good metabolic activity (based on an incorporation of sulphate into newly synthesized glycosaminoglycans (GAGs) at 70% of that of fresh control cartilage). The first experiments were carried out using a Dow Corning oil bath for step-wise cooling, and manual transfer of the samples to increase CPA sufficiently to suppress ice formation at the set temperatures; thereafter, a controlled rate Planer freezer was used to achieve continuously lowering temperatures which could also be held at selected low temperatures to allow for CPA equilibration. By applying continuous stirring throughout the process, a significant increase of GAG synthesis to 87% of the corresponding fresh control values was reached. Preservation of human knee joint cartilage resulted in GAG synthesis of 70% and ovine knee joint osteochondral dowels of 60% of fresh controls after this LT approach.

For optimisation, David Pegg in collaboration with Planer plc (a company specializing in cryogenic engineering), established an automatic pump and stirring system for use within a controlled rate cryo-cooler, which constantly increased the CPA concentration whilst the temperature was decreased. For the warming process normally the reverse protocol is used by constantly increasing the temperature whilst the CPA concentration is reduced by dilution.

APPRAISAL OF THE MANUAL APPROACH TO LT FOR LABORATORY STUDIES

Manual Liquidus Tracking holds some logistical advantages in that it does not require complex equipment, and pre-set CPA solutions can be prepared before the start of the procedure. It can allow small batch vitrification for moderate volumes (in the region of 2 – 10 ml), and can help in testing various LT conditions in parallel within one experiment (e.g. buffers, AntiFreeze Proteins, antioxidants, CPAs) which might be used to optimize LT. However, it is demanding and requires close attention to the various steps within LT, is limited by some cryo-physical properties, and thus is liable to higher operator-induced variabilities. We have defined three test set-ups which can be used to perform manual Liquidus Tracking.

LT Set-up 1- sample transfer between different containers during cooling.

Set-up 1 corresponds to the manual LT approach used by Farrant (6), Elford (3) and Pegg (14). A series of pre-set low temperature baths can be used to cool the samples step-wise

with the appropriate CPA solutions loaded into containers at each temperature to avoid ice nucleation. The sample to be vitrified is transferred from one sample container to the next, each with a higher CPA concentration and at a lower temperature, with a hold-period at each time to allow sufficient CPA penetration (Although objective evidence of this time period is currently lacking, a general range can be gleaned from previous studies (14). This set-up is only suitable for tissue pieces, which can be transferred by using forceps or encapsulated cells of a unit size allowing them to be trapped by a suitable cell strainer in which the sample can be transferred. The necessary increase in CPA can be calculated, and the CPA concentration can be measured using a refractometer to ensure the correct value has been reached. (19) – e.g. see Figure 2.

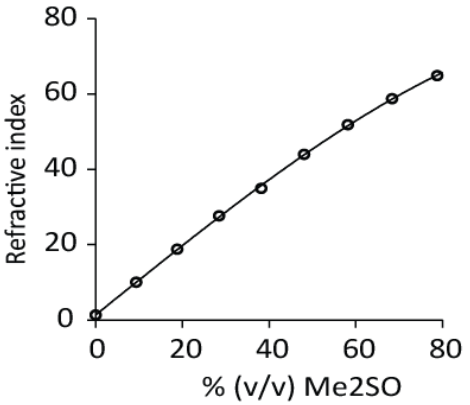

Figure 2. The refractive index of an aqueous solution (Me_2SO in 1x PBS at 20°C) as a function of increasing CPA concentrations. By using the standard curve (R=0.9993) the CPA concentration of a sample can be determined.

In contrast, when using a controlled rate freezer, all samples and solutions can be placed into the freezer chamber at the start of the run at fixed temperatures the cooling chamber has to be opened to transfer the sample to the next higher CPA solution. Samples can be processed for example in 6-well plates. Possible temperatures, at which the transfer can be carried out when using Me_2SO are -5°C (from 20% to 30% v/v CPA), -10°C (from 30% to 40% CPA), -20°C (from 40% to 50% CPA), -30°C (from 50% to 60% CPA) and -40°C (from 60% to 70% CPA) using v/v Me_2SO. The transfer temperature includes an offset of approximately +30% from the actual calculated T_m of the sample to prevent any potential freezing. A schematic of set-up 1 is shown in Figure 3 and Table 1. The lowest temperature which can be chosen for the transfer is determined by the CPA concentration of the starting plate as it contains the lowest CPA concentration. Subject to the volume of CPA mix used for each sample step, the cooling rate in the sample (as opposed to the chamber) can become very slow when the sample temperature gets close to the next hold-step/transfer temperature. This was detected by placing thermocouples into dummy wells. To overcome this, the freezer temperature can be set a few degrees below the desired transfer temperature; however, this risks potential ice nucleation if the process drifts too far away from the liquidus curve. This risk can be mitigated by recording temperatures from the thermocouples in the dummy wells, and monitoring the progress of cooling. In our case the freezer holding temperature was set -10 degrees below the planned transfer temperature to allow sufficient cooling with a holding time of 10 minutes and a freezer cooling rate of -2°C/min. This was used to allow approximately 30 minutes between each stepwise CPA increase. The sample volume was 8 ml per well for a 6-well plate set-up. The incubation time was chosen on the basis of Pegg's work on articular cartilage (20). Cell densities of alginate encapsulated HepG2 liver cells are lower than those for articular cartilage and therefore it was assumed that sufficient time for CPA penetration was allowed. To process all samples at the same freezer temperature, the freezer had to be set on hold-in our studies for 10 minutes. The hold depends on how fast the transfer temperature can be reached, how many samples have to be processed and how much time should be given for CPA penetration. Depending on the experiment 6, 12 or 24 well plates may be used, but CPA diffusion and heat transfer parameters might vary and should be tested.

One disadvantage of this approach can be a solution carry-over effect which occurs each time the samples are transferred in the cell strainer to the next higher CPA concentration and results from surface adherence of residual CPA solution from the lower concentration on the cell strainer or accumulated cell capsules tissue pieces. This results in an uncontrolled and successively increasing dilution of the CPA solutions which might cause sample-to-sample variability, and at worst might lead to ice formation as lower temperatures are reached. In addition, manual moving of the samples during the transfer can cause inadvertent and variable

warming, depending on how fast the sample can be moved to the next CPA well.

When using a controlled rate freezer (e.g. cooled by circulating vapour generated by the injection of liquid nitrogen), it should be checked that all positions within the freezer (close to circulation fan) and within the plate (middle or corner) provide the same cooling rate, as inhomogeneous heat transfer between the wells can lead to sample-to-sample variability; random ice formation in some samples was visually noted in our experiments.

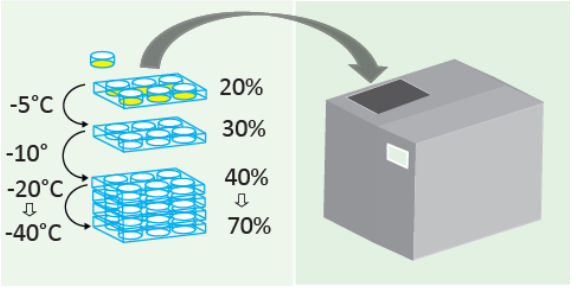

Figure 3. A schematic showing samples (in cell strainers) being transferred between 6 well plates with increasing CPA concentration (e.g. 20% 30%, 40%, 50%, 60% and 70% Me_2SO) as the cooling proceeds. All plates are placed into the freezer at the beginning of the run. Possible temperatures at which the change can be carried out when using Me_2SO are -5°C (from 20% to 30%), -10°C (from 30% to 40%), -20°C (from 40% to 50%), -30°C (from 50% to 60%) and -40°C (from 60% to 70%) (v/v) Me_2SO.

Table 1. Scheme of possible temperatures at which the transfer can be carried out when using Me_2SO.

% (v/v) Me_2SO from:	transfer at:	melting point of Me_2SO at:	freezer holding temperature
20 to 30	-5°C	20%: -8°C	-15°C
30 to 40	-10°C	30%: -15°C	-20°C
40 to 50	-20°C	40%: -30°C	-30°C
50 to 60	-30°C*	50%: -50°C	-40°C
60 to 70	-40°C*	N/A	-50°C

*transfer temperatures were determined empirically, as viscosities at colder temperatures are too difficult to handle. N/A states for non-applicable.

LT Set-up 2 – adding volumes of increasingly higher CPA solutions in a single container

When using set-up 2 the sample remains in the same sample container throughout the LT vitrification procedure. For each incremental cooling step, the CPA concentration is increased by the addition of a higher concentration CPA solution to achieve the required LT threshold concentration. For example to increase the concentration by increments of 10%, an equal volume of a 20% absolute higher concentrated CPA solution can be added. However, one noted disadvantage is the increase in total CPA volume as each additional step is made. This risk can be mitigated before the next CPA addition by reducing the sample volume e.g by pipetting off half the volume once the cell material has settled. Without this supernatant reduction step, the volume might exceed the original tube capacity but will certainly exceed the volume that can be manually mixed at low temperatures, without drastically increasing the temperature of the sample, a result of the mixing procedure itself. Besides the mixing difficulties, the cooling rate profiles and properties of the sample would change dramatically over the incremental steps of the protocol. It can be recommended from personal observations that for these reasons, the volume to be mixed should not exceed more than 6 ml in the final step. A schematic of set-up 2 is shown in Figure 4.

As the volume has to be reduced, this set-up is only feasible for sample material that will accumulate at the bottom of the tube by gravity (in the time given until the next CPA addition step). Otherwise special filter tips/ transfer processes have to be developed which may be quite cumbersome (assuming that the tissues or entrapped cells sediment by gravity), and will again interfere with mixing. At the beginning of the run enough CPA solutions of e.g. 40% 50%, 60%, 70% and 80% CPA have to be stored within the freezer to be cooled. The volume of each solution should be similar to the sample volume to assure corresponding cooling rates.

The advantage of this approach is that the sample remains within the same sample holder during the whole LT process and the sample is mixed during each step. The cooling rate will be similar to the cooling rate of the freezer chamber, especially when microfuge tubes and volumes below 1 ml are used, and therefore the hold temperatures of the cooling profile can be equal to the desired transfer temperature. The time the freezer has to be set on hold will therefore mainly depend on number of samples within the run, and CPA diffusion parameters at low temperatures (although as stated already, little objective information exists for this). To allow a diffusion period of approximately 30 minutes and to provide a similar cooling profile as for set-up 1, the holding temperature was first set at the average temperature between the

starting and the transfer temperature for 15 minutes. Then an additional holding step was set at 5°C below the transfer temperature for 7 minutes to reach the required temperature and to process the samples. As for set-up 1, a freezer cooling rate of -2°C/min was chosen.

The primary disadvantage of set-up 2 is the difficulty of maintaining the temperature whilst CPA concentration is changed. As the freezer chamber is opened to allow manual access, the temperature inside the freezer may deviate rapidly, affecting the temperature of the solutions kept inside the freezer. Extracting and adding half of the sample volume and the mixing process takes time and will impact the sample temperature depending on its volume, with small volumes being more easily subject to temperature fluctuation. For our feasibility study, 2 ml microfuge tubes were used with a maximum volume of 1.5 ml. During the CPA addition processes a temperature increase of up to +20°C was measured by thermocouples placed in sample tubes. A second disadvantage might be the numbers of tubes with different CPA solutions that have to be kept in the freezer which can be complicated when comparing different conditions within the same run.

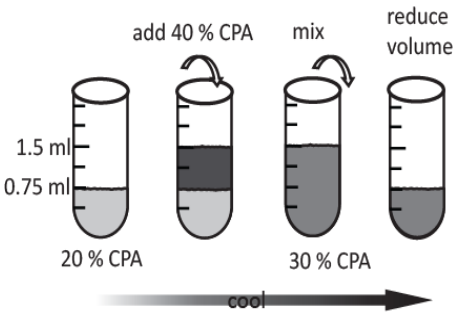

Figure 4. A schematic of set-up 2. CPA concentration by increments of 10% is increased by adding an equal amount of a 20% higher concentrated CPA solution. After mixing, the volume is reduced. The same transfer temperatures as for set-up 1 (Table 1) can be used.

LT Set-up 3 – based on use of one highly concentrated CPA solution to provide required concentration increase.

Set-up 3 is a variant of LT set-up 2 whereby only one highly concentrated CPA solutions is used to make the stepwise required CPA concentration. Since the final steps of LT require solutions in the range of 60% CPA, an extremely concentrated CPA (80% v/v) is needed to achieve this goal. One advantage of set-up 3 is that only smaller volumes need to be added at each step, avoiding the problem of accumulating supernatant volumes, and therefore the sample volume does not have to be reduced each time as for set-up 2. Several other advantages can be outlined for this approach: as only one solution has to be added the accuracy of achieving the target CPA improves; there is no loss of cell material and the process is faster which means less sample-to-sample variability due to potential temperature variation. The average temperature increase during each CPA addition and mixing step in our experiments was determined to be approximately +11°C. Even so, heat transfer for the steps in set-up 3 is slightly different as volumes change, but nevertheless the same holding time and temperatures were used on a pragmatic basis. A schematic of set-up 3 is shown in Figure 5 and Table 2.

A disadvantage is that highly concentrated CPA is added to the sample at relatively high temperatures which could be a problem for cells that are especially prone to osmotic stress and CPA toxicity if the mixing is not quick enough. As a compromise two different high concentrated solutions could be used, e.g. with a concentration of 60% for the first steps and 80% final increase. Even though the final volume is limited to 5-6 ml, mixing still might become difficult during the last steps depending on the CPAs used and their low-temperature viscosities.

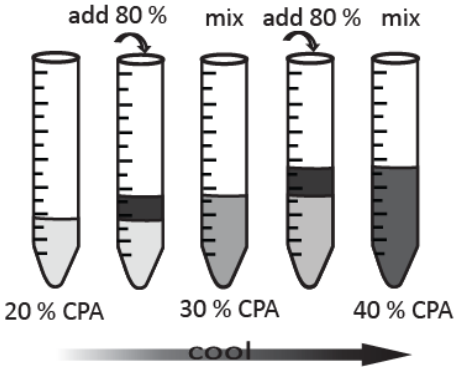

Figure 5. A schematic of set-up 3. One highly concentrated CPA solutions (80%) increases the CPA concentration of the sample. Smaller volumes have to be added to increase the sample CPA concentration so sample volume does not have to be reduced after each addition as for set-up 2.

Table 2. Amount of 80% v/v CPA that has to be added to increase the sample CPA concentration by increments of 10%

Initial CPA (%)	Initial Vol. (µl)	Vol. (µl) of 80% CPA to add	Target CPA (%)
20	750	150	30
30	900	225	40
40	1125	375	50
50	1500	750	60
60	2250	2250	70
final vol.	4500		

Formula to determine the amount of 80% CPA to be added: B=(A*c-A*a)/(b-c) with B=vol. of 80% CPA to add, A=start vol., c=final CPA concentration, a=start CPA concentration, b=80% CPA

WARMING

The equally important principle of LT is that a reverse process is essential, where the high CPA concentrations are reduced, at intermediate subzero temperatures sufficient to be above the liquidus curve, again in a progressive or step-wise manner, as the samples progress back up the temperature scale. If only one controlled rate freezer is available it is recommended to use only two different temperatures steps. Differently concentrated CPA solutions cannot be cooled down within the same freezer if the freezer temperature is kept close to the melting temperature of the sample, otherwise they will freeze. This may be overcome if multiple low temperature baths are available. For example, according to the liquidus curve a sample of 50% Me_2SO (v/v) should be kept at approximately -39°C. A 40% Me_2SO solution for the dilution has to be kept above -23°C and a solution of 30% above -14°C. The temperature closest to the sample liquidus curve that can be used for the first dilution would therefore be -23°C; a temperature at which the 30% CPA solution would already nucleate. This can be overcome by raising the freezer temperature after each dilution step before placing the next more dilute solution into the freezer. However it will take time for the dilution solution to cool down to the freezer temperature from approximately 0°C when kept on ice. Therefore the warming process might take several hours resulting in longer exposure times to CPA and cold storage. Given the limits of manual practicalities and the need to reduce the potentially-toxic levels of CPA, a two-step procedure might therefore be the better choice. For example the freezer could be set to -20°C and all dilution steps to 40% CPA (depending on the CPAs used) could be done at that temperature. The second step could then be done on ice with a CPA decrease to directly 0%, advisably by using an osmotic buffer. To reduce the sample CPA concentration, more dilute CPA can either be added to the same tube or the sample can be poured through a cell strainer and then transferred to a less concentrated CPA solution. Adding CPA to the sample means substantially increasing the sample volume and this has to be reduced either by using a filter system (cell strainer or pipette tips with filter) or by gravitation and removal of the supernatant. CPA solutions can be very viscous at low temperatures and samples may need to be diluted before using any type of filter system. Also tissue pieces or encapsulated cell beads might stick to the tube wall when transferring to a cell strainer, resulting in loss of starting material. A wash step with CPA solution at subzero temperatures can be performed to mitigate this. Depending on how susceptible cells are to osmotic stress and CPA toxicity, either the faster method or the method that maintains the sample temperature closest to the melting point can be chosen.

FEASABILITY STUDIES – RESULTS CONCERNING MANUAL LIQUIDUS TRACKING

In an initial experiment we investigated the concept that CPA toxicity can indeed be reduced at subzero temperatures when applying the highly concentrated CPA needed for LT. Therefore alginate encapsulated liver cells, cultured for 4-5 days were incubated at 37°C (incubator), 20°C (room temperature), 4°C (fridge), -10°C and -20°C (Planer Kryo 10 freezer) for 40 minutes in increasing concentrations of Me_2SO (Figure 6). Beads incubated at -10°C and -20°C were pre-incubated for 1 min at 0.5°C (ice water) in 20% (v/v) and 30% (v/v) Me_2SO, respectively. This was done to prevent immediate intra- and extracellular freezing of the cells once the sample was exposed to subzero temperatures.

After the incubation, beads were washed with 1xPBS (Mg^{2+}, Ca^{2+}) and viability was measured as described previously (12). The lower the incubation temperature, the higher was the remaining viability. Up to 50% (v/v) Me_2SO was well tolerated when alginate encapsulated HepG2 cells were incubated at -20°C but dropped to 0% for all higher temperatures between -10°C and +37°C.

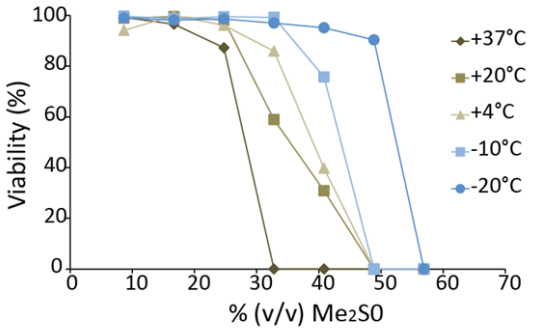

Figure 6. Encapsulated cells were incubated at increasing Me_2SO concentrations at decreasing temperatures, for 40 minutes. Up to 50% (v/v) Me_2SO was well tolerated when alginate encapsulated HepG2 cells were incubated at -20°C, showing that Me_2SO toxicity (osmotic and chemical) is reduced at lower temperatures. Viability dropped to 0% when a certain Me_2SO concentration was reached. Viability was assessed 24h after rewarming using fluorescent vital dyes, fluorescein diacetate and propidium iodide (11).

In order to investigate at which step viability was mostly decreased during the LT procedure, step-by-step LT experiments were carried out (Figure 7). All three set-ups; 1, 2 and 3 (described above) were tested. The steps were chosen to cover the incremental increase in CPA concentration and cooling, down to the point where the vitreous transformation would be predicted (see Figure 7). Some samples were removed at each stage just before the next addition of Me_2SO, and rewarmed for viability measurements, whilst the remaining cohort stayed in the controlled rate freezer as the LT run progressed further. In brief, (1) at -5°C, (2) the 20% Me_2SO solution with alginate encapsulated beads was increased to 30% Me_2SO with mixing; this was then repeated (3) at -10°C with increase to 40% Me_2SO, (4) at -20°C with increase to 50% Me_2SO, (5) at -30°C to 60% Me_2SO and (6) at -40°C to 70% v/v Me_2SO. Between each CPA addition, 30 minutes were allowed for cooling and CPA change-(see set-ups 1-3). In the final step (7), two of the two remaining samples were slowly cooled down to -60°C. One sample was warmed up immediately; the other one was directly added to the vapour phase of liquid nitrogen where it was stored for 3-7 days before warming. For the complete LT pathway, the time taken to achieve the vitrified state was about 150 minutes. A simplified reversal of the LT protocol was used to warm samples after cooling. To avoid osmotic injury during dilution, the Me_2SO increase per step was limited to a 30% (v/v) concentration reduction. Samples with a Me_2SO concentration of 20-30% (v/v) were placed directly into (1) ice-cold 1xPBS (Sigma, Mg^{2+}, Ca^{2+}) for 10 minutes, then washed several times with 1xPBS, transferred to complete media and incubated at 37°C. Samples with a final concentration of 40-50% (v/v) Me_2SO were placed into ice-cold 20% (v/v) Me_2SO for 10 minutes and were then processed as described in (1). Samples at 60% (v/v) Me_2SO and samples at 70% (w/w) Me_2SO at -60°C were first placed into 40% (v/v) Me_2SO at -20°C for 15 minutes, then into ice-cold 20% (v/v) Me_2SO for 10 minutes and were then processed as described in (1). Lastly, vitrified samples stored at -160°C were transferred to -80°C for 20 minutes and were then processed equally to those samples simply cooled to -60°C. For vitrified samples, the complete process of reversed LT took approximately 1 hour. From Figure 7 it is clear that viability was decreased after each LT step, and thus the results did not indicate a specific concentration at which viability was primarily reduced. The data suggest that viability is mainly reduced between 30% and 60% (v/v) Me_2SO but remains constant between 60% and 70% (v/v) Me_2SO, regardless of whether samples are cooled down to -60°C and immediately re-warmed or stored at -160°C. The experiments showed that cell viabilities can be ~30% after achieving the vitrified state and reversal via LT. To our knowledge, this is the first report of viable cell recoveries using LT for encapsulated cell organoids. The fact that approximately two thirds of the functional cell mass was lost during LT may seem disappointing, but on the other hand, the results indicate that a significant proportion of mammalian cells can withstand controlled vitrification by LT. It may be possible to increase the survival by attention to detail in each of the progressive steps of the overall LT process.

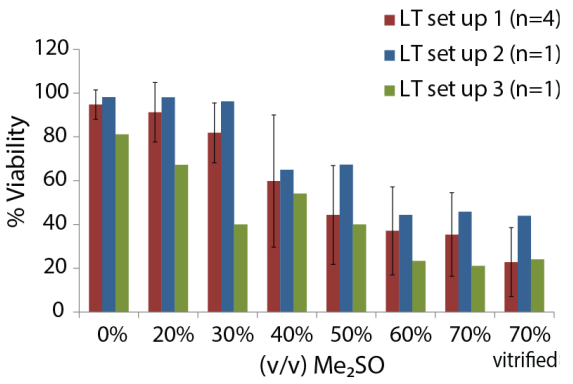

Figure 7. LT step-by-step: To determine at which step viability is lost during the LT procedure a step-by-step LT set-up was carried out. Therefore one sample was taken out of the Planer freezer before the Me_2SO concentration was increased and temperature further reduced for the remaining samples. All three set-ups; 1, 2 and 3 were tested. Considerable variability is seen between runs. Nevertheless viability dropped mostly between 30% and 60% Me_2SO (v/v) (except for set-up 2). Viability after incubation in 70% (v/v) Me_2SO is approximately 30% regardless of whether samples were cooled to -60°C or cooled and stored at -160°C. Viability was assessed 24h after rewarming using fluorescent vital dyes, fluorescein diacetate and propidium iodide (11). Data for set-up 1 was n=4 +/-SD.

Preliminary indications for improving laboratory translation for manual LT: - better control of temperature profiles.

Set-up number 3 has shown to be the most practical in our hands. Nevertheless heat fluctuation during the CPA addition process was observed. The increase in sample temperature was especially large at lower temperatures. This was mainly caused by a prolonged mixing procedure due to increasing sample viscosity and the larger volumes to be mixed. One way to provide a more reliable process during manual LT could be by using a 'thermal buffer- jacket - TB' – by placing each sample container (15 ml centrifuge tube) within a 70% ethanol solution held in a 50 ml centrifuge tube. The following was considered necessary for a useful TB system: the solution used for TB should not freeze during the cooling or warming process as this would affect the cooling rate of the LT sample itself due to altered heat capacities. Also the TB solution has to remain liquid so that the LT sample tube can be taken out from the TB at the end of the run and stored in liquid nitrogen. Additionally, the TB system must be as symmetrical as possible to allow a homogenous cooling profile throughout the entire volume. The toxicity of the TB carrier solution should also be as low as possible for handling purposes, and the TB should be of a size that can be fitted within a controlled rate freezer (e.g. used here, the Planer Kryo -10 freezer). The cooling rate of the sample during the LT steps should be affected as little as possible, while the TB isolation should maintain the same temperature for several minutes in an altered environment until the CPA change has been processed. Other combinations of TB carrier solutions and tubes with better thermal buffering abilities might be used. Nevertheless this will lead to lower sample cooling rates causing longer runs and therefore longer exposure times to CPAs and cold temperatures. By using the described TB isolation system the temperature fluctuation was reduced from a maximum of +18°C (CPA addition at -35°C) to less than + 5°C (n=3).

Additionally, handling variability can be compensated for by testing a higher number of samples. However the maximum number of samples that can be processed during one LT run has to be limited to maintain similar sample warming and incubation times for all the processed samples. Thus, in our experience, in the present manual LT feasibility it was found that the maximum number of samples per experiment was 8, , all of which could be housed in the chamber of a Kryo 10 cooler and processed within 2 minutes, including CPA addition and mixing.

DISCUSSION

The fundamental basis of LT theory encompasses the concept that CPA toxicity can be reduced if the temperature is decreased, such that concentrations high enough to lead to vitrification can be achieved step-by-step at lower temperatures. This has been shown in previous work by Matheny in 1968 (13) who incubated rabbit atria in different Me_2SO concentrations at decreasing temperatures. The effect is also reflected by reduced drug efficiency at lower body temperature, which has been widely studied by Weihe in 1973 (21). Reduced toxicity and decreased drug efficiency can be explained by decreased enzyme activity, which for mammalian cells is highest at ~37°C, and also a result of decreased reaction kinetics in general, which includes reduced chemical and/or osmotic stresses. This Liquidus Tracking (LT)

principle was confirmed in our current work (Figure 6) whereby incubating alginate encapsulated liver cells in increasing concentrations of Me_2SO at temperatures between 37°C and -20°C showed less injury at lower temperatures. Nevertheless reduced toxicity at lower temperatures does not assure effective vitrification, since sufficient CPA has to penetrate into the cells to avoid ice nucleation before the temperature is further decreased. It is possible that in these experiments cells incubated at -10°C or -20°C did not reach the same intra-cellular Me_2SO concentration as cells incubated at higher temperatures. For an optimized liquidus tracking procedure it is therefore necessary to compromise between longer exposure time and higher exposure temperature to achieve sufficient CPA penetration, and shorter exposure time at lower temperatures for minimum toxicity.

Three manual LT approaches were tested and compared. Advantages and disadvantages have been addressed in section 3. All three methods showed a constant small but progressive decrease in viability after each Me_2SO concentration increase. The data (Figure 7) suggests that viability was mainly reduced between the steps from 30% to 60% (v/v) Me_2SO (except set-up 3) but thereafter remained constant between 60% and 70% (v/v) Me_2SO, regardless of whether samples were cooled down to -60°C and immediately re-warmed or vitrified and stored at -160°C. An explication for this could be that CPA concentrations up to 30% (v/v) Me_2SO are well tolerated for over 40 minutes when kept at 0.5°C (Figure 6). Concentrations of 60% and 70% (v/v) Me_2SO are reached at very low temperatures (<-25°C) and it could be hypothesized that at these low temperatures, the Me_2SO influx is limited so that full intracellular Me_2SO concentrations of 60-70% (v/v) might not be achieved (which could explain higher consistency between these steps). In this respect, cells that survived the LT process until the final increase to 70% (v/v) Me_2SO seem to have achieved sufficient Me_2SO penetration to be vitrified. This could of course also be brought about by the high viscosities of the Me_2SO mixture acting to kinetically inhibit ice crystal formation, and the parallel extreme osmotic dehydration of the cells which also prevented organisation of any residual water molecules into ice nuclei. Further work will be needed to investigate these possibilities. However, morphologically it was possible to visibly discern when ice nucleation had taken place in individual samples which had not been appropriately incubated in the early stages of the feasibility study, particularly using these large volumes. Thus we feel confident that we managed to reproduce essential LT in our selected protocols.

High variability between experiments can be partially explained by temperature profile variations and CPA concentration differences between samples resulting from the different manual approaches to achieving LT. Inter-sample temperature differences for set-up 1 may have been caused by the sample position within the freezer, the differing thermal masses of different LT receptacles – e.g. position in the 6-well plate (corner or middle) and time taken to change the CPA concentration. Divergent CPA concentrations across the LT steps could have resulted from pipetting difficulties with the highly viscous higher CPA solutions, and 'carry over' effects in those experiments where samples were physically moved between different Me_2SO mixtures. In our experience, set-up 3 was the most easily and consistently achieved LT protocol when considering all the logistical hurdles. Higher post-warming viabilities and performance can be expected for further improvements in the manual LT method. Reduced temperature fluctuations (by using thermal buffering of individual samples) and improved CPA addition and mixing steps (by seeking better mixers) should help to make the change.

CONCLUSION

LT by manual protocols has been shown to be a complex and lengthy procedure, but it does allow larger volumes to be stored in the vitrified state when only slow cooling rates can be achieved. For example, given the number of samples which could be handled in our set-up 3 (up to 8 tubes with 5 ml in each) a biomass of about 40 ml could be vitrified in one run, which is far higher than could be considered for the same materials if traditional vitrification methods were applied (samples <100 µl plus and ultra-rapid cooling). Some of the complications that were observed for manual LT may not apply for an optimized automatic approach, which would minimize inhomogeneous Me_2SO concentrations and temperature fluctuations during step Me_2SO changes. Moreover the Me_2SO concentration will potentially be

increased in smaller steps and therefore toxicity and osmotic effects should be further reduced. In addition, by its nature, manual liquidus tracking is complicated and can result in high variability. Nevertheless, this approach offers the possibility of testing several conditions at the same time and could therefore be used to pre-test different LT factors before being taken forward to automatic LT. Using a combination of penetrating and non-penetrating CPAs, instead of Me_2SO as a sole reagent, combined with the improvements in manual set-ups, should offer further improvements in post-warming viable cell recoveries.

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