Dimethyl sulfoxide for cryopreservation of alginate encapsulated liver cell spheroids in bioartificial liver support; assessments of cryoprotectant toxicity tolerance and dilution strategies.

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Abstract

The Bioartificial Liver (BAL) is an extra-corporeal liver support designed to support the function of the Liver in patients with impaired liver function. The BAL biomass consists of alginate encapsulated liver spheroids (AELS). To facilitate rapid delivery of a BAL to patients the AELS are cryopreserved using a DMSO-containing cryoprotectant solution.

This study assesses toxicity of DMSO in AELS at concentrations and temperatures relevant to the cryopreservation and recovery process of a cellular biomass. Additionally, develops a process to remove DMSO from AELS before delivery of cell product to patients.

Exposure of AELS to DMSO, at a concentration of 12% (v/v) for 10 minutes did not have a negative effect on the viability of the AELS up to 24 hours after exposure, irrespective of the exposure temperature between 37 C and 0 C. Evidence of toxicity was only seen with exposure to 40% (v/v) DMSO, which was more notable at warm temperatures.

Post-Thaw removal of DMSO was measured by determining the DMSO concentration of the post-thaw washes using refractometry. Washing AELS 3 times in tapering concentrations of Glucose

supplemented DMEM at an AELS:wash ratio of 1:2 was sufficient to reduce DMSO to undetectable levels (<1%).

The study demonstrated that the thawing method minimised DMSO toxicity to the BAL biomass, and the post-thaw washing protocol successfully removed all the DMSO present in the cryopreserved BAL. Thereby enabling effective cryopreservation of the BAL for future clinical translation.

Keywords

DMSO, DMSO Toxicity, Cryoprotectant Toxicity, BioArtificial Liver, Tissue engineering, Liver Failure

Introduction

Liver disease kills over 10000 people a year in UK. Liver failure is a rapid onset disease and can lead to death within a few days. There is a need for a therapy to support and supplement the function of the liver to allow time for a transplant to become available, or the patient's liver to regenerate itself. We have developed a BioArtificial Liver (BAL) based on liver cell spheroids encapsulated in alginate. However, responsive manufacture of the BAL is not logistically feasible and cryopreservation is necessary for 'off the shelf' availability.

Dimethyl Sulfoxide (DMSO) was identified as a cryoprotective agent (CPA) in 1959 when it was used to ameliorate freezing induced injury in bull sperm by Lovelock and Bishop [13]. Since then it has become widely used as a CPA in the preservation of cells and tissues. However, despite its cryoprotective properties DMSO is considered to be cytotoxic at physiological temperatures. It has been shown to have adverse effects on the plasma membrane [21,23] and damaging effects on the cytoskeleton [11,22], even causing apoptosis [7]. Therefore, successful cryopreservation strategies minimise the exposure of cells to DMSO at higher temperatures. DMSO loading and removal is generally carried out at <5°C. Moreover, there is now a regulatory push to remove DMSO from cryopreservation protocols, wherever practical.

Cellular therapies cryopreserved with DMSO could produce side effects and complications in patients if care is not taken to remove all the cryoprotectant prior to delivering the therapy. Delivery of cell products to patients without removal of DMSO has been linked to a wide range of adverse effects such as neurotoxicity [8,16], cardiac arrhythmia [9] and hepatic dysfunction [20,24,26]

The biological component of the BAL consists of a 2.5L biomass of alginate encapsulated liver spheroids (AELS) (~70 billion cells). To prevent toxicity the biomass is cooled before addition of a DMSO-containing cryoprotectant solution (CPS) and the BAL is cryopreserved. However, the cryopreserved BAL is thawed rapidly at 37°C. Due to its size and geometry parts of the BAL biomass maybe exposed to DMSO at temperatures where the CPA may be potentially toxic, before the entire biomass has thawed. We modelled these transient exposures to DMSO over a range of temperatures on AELS to assess any potential toxicity. In routine cryopreservation of small volumes (1 – 2mls), rewarming times, post-thaw handling and dilution can all be completed in about 30 minutes. For large volumes (>2 L), such as the BAL , these times can be up to 60 -90 minutes.

Of equal importance, delivery of significant concentrations of DMSO to the patient is also undesirable, so a dilution strategy to remove DMSO effectively from the cell therapy product is required. We have developed a washing protocol that removes DMSO from the biomass without causing undue osmotic stress to the AELS as integrated units, which can be assessed morphologically. To achieve these aims a method was developed to measure removal of DMSO using refractometry.

Therefore, the aims of the current study were three-fold; (1) to investigate the potential for CPA toxicity to AELS by viability measurements given the exposure times which correlate to handling steps in our warming and dilution protocols; (2) to measure the stability of the alginate bead component of AELS during DMSO exposure and dilution; and (3) to quantify the effectivity of the sequential dilution steps in reducing the concentrations of DMSO for protocol optimisation.

Materials & Methods

Cell Culture

HepG2 cells (ECACC Wiltshire) were cultured in modified α-MEM (GE Healthcare) supplemented with 10% Fetal Bovine Serum (FBS) (GIbco), 1% Penicillin/Streptomycin (Gibco) and 0.5% Amphotericin (Gibco), to grow sufficient cells in monolayer to seed subsequent 3D culture. Media was changed every 2-3 days.

After encapsulation the AELS were cultured in media containing Fresh Frozen Plasma (FFP) in place of FBS.

Cell Encapsulation

HepG2 cells were encapsulated as previously described [5]. Briefly, HepG2 cells were trypsinised and resuspended in culture medium at 4x10^6 cells/mL, mixed 1:1 with 2% alginate to achieve a 1% alginate and cell solution at 2x10^6 cells/ml. 1.5% glass beads added as a density modifier. Using the JetCutter system (GeniaLab), solution was passed through a nozzle and the stream cut using a wire-cutting disk, droplets fell into a crosslinking 0.204M $CaCl_2$ solution for 5 minutes. Encapsulated cells were washed three times with culture media to remove excess calcium and then cultured as required.

DMSO toxicity assessment

AELS exposure to DMSO was modelled to mimic the potential exposure during the thawing process. AELS were cultured in 6 well plates for 12 days. AELS were exposed to a final concentration of 12% DMSO and 38% viaspan (Belzer UW, Bridge to Life), which is our chosen excipient for CPA delivery, for 10 minutes at 0°C, 20°C or 37°C, with 40% DMSO as a positive control for damage. The time period (10 minutes) was chosen as an indicative time noted from our previous studies to handle AELS at the end of thawing and move them to the dilution process [10]. After treatment, AELS were exposed to 3 subsequent media washes at temperatures of 4°C : 1M glucose α-MEM, 0.5M glucose

α-MEM, and unsupplemented α-MEM using protocols previously developed to use the addition of glucose to try and avoid osmotic shock during DMSO removal [3]. Treated and washed AELS were returned to culture medium and cultured for a further 24 hours. Viable cell number was assessed throughout the experiment.

Viability assessment

Metabolic viability was assessed by vital dye staining using Fluorescein Diacetate (FDA) [Sigma #F7378] and Propidium Iodide (PI) [Sigma #P4864] as previously described [14]. Briefly 250µL of AELS were washed twice with PBS^{+Ca+Mg}, stained for 90 seconds with 0.0128 mg/mL fluorescein diacetate (FDA) and 0.0256 mg/mL propidium iodide (PI), washed again with PBS^{+Ca+Mg} and imaged using an inverted Fluorescence microscope (Nikon), Quantification was performed with NIS elements software (Nikon)

Nuclei quantification

250µL of AELS were washed twice with Hank's Buffered Saline Solution and cells were liberated from the alginate using 16mM Ethylenediaminetetraacetic acid (EDTA), centrifuged at 14000 g, and the pellet was resuspended in PBS^{-Ca-Mg} and disaggregated using a 21G needle followed by automated nuclei quantification using Nucleocounter NC-100 (Chemometec) according to manufacturer's instructions.

Refractive index to measure DMSO removal

Refractive index (RFI) is a dimensionless value calculated from the ratio of the speed of light through a vacuum and a medium of interest. The RFI of a solution is affected by its density; as the mass of DMSO in a solution changes so does the density and therefore the RFI [12]. The efficacy of using RFI to measure DMSO in cells was tested on the cell free scaffolds, alginate beads without cell spheroids. Standard curves of DMSO in each washing solution were constructed. To test CPS removal 2mL of cell-free alginate beads were loaded with CPS in a 1:1 ratio and incubated at room temperature for 5 minutes to allow penetration of CPS into beads. The beads were drained through a 100µm cell

strainer and the supernatant retained. The beads were subsequently washed with reducing concentrations of glucose supplemented DMEM (1M → 0.5M → 50mM → 50mM) in three different bead:wash ratios (1:1, 1:2, 1:3). The supernatant from each wash was collected and RFI was measured, determining the concentration of DMSO with reference to a standard curve.

This washing protocol was tested on cryopreserved AELS. Due to the cellular component the same standard curves could not be used to accurately measure DMSO concentration of these washes. The standard curves had to be constructed using washes that had previously been filtered through AELS, thus preventing any cell debris from impacting the RFI.

Morphological assessment of the AELS microbead units during CPA exposure and dilution

Alginate bead shape and dimensions were analysed through microscopy and image analysis. 0.3 mL of AELS were sampled, transferred to a microscope slide, cover slipped and imaged at 4x magnification. At least 10 photos were taken per condition. AELS were not washed prior to imaging as washing could affect the size and shape of the beads due to osmotic changes. The EqDiameter and Circularity of beads was measured using NIS-Elements Imaging software.

Statistical Analyses

Statistical analysis was conducted in R (R Core Team, 2019). Significance was tested using students t-test and threshold was significance was set at $p<0.05$.

Results

There was no immediate effect on functional viability of AELS after exposure to 12% DMSO at either 4°C, 24°C or 37°C. Additionally 12% DMSO also had no effect on viable cell number. After 24 hours in culture there was no difference in viability or viable cell number in AELS exposed to 12% DMSO and washed free from the CPA, regardless of temperature. On the other hand, exposure to 40% DMSO at

RT resulted in immediate loss of AELS functional viability, accompanied by a loss in viable cell number **Error! Reference source not found.**. However, these were for short exposure times (10 minutes) which can result from warming post-thaw handling and dilution; for large volume cryopreservation the exposure times can be up to 5 times longer.

Residual DMSO concentrations were measured using refractometry of the collected wash volumes. Already by 1 wash, the CPA concentrations had been reduced by more than 50% using either 1:1. 1:2 or 1:3 dilution volumes. There was a trend for greater dilution efficiency using 1:3 but the DMSO concentration was still at some 30% of the staring value. DMSO was undetectable by refractometry in the AELS samples after 3 washes at a 1:2 ratio and 2 washes at a 1:3 ratio. At a 1:1 ratio, trace amounts of DMSO were still detectable (at a percentage of 1% (w/v)) even after 4 washes Error! Reference source not found..

Size analyses of the alginate beads through the steps of the protocol are shown in Figure 3. During cooling of AELS in readiness for exposure to DMSO, there was a trend (non-significant) to a reduction in bead diameter. This could reflect a re-arrangement in alginate polymer structure and/or alginate interactions with the cell organoids, their extracellular matrix and free water in the aqueous system. After addition of chilled DMSO solution, bead sizes assumed a statistically significant reduction to about 85% of starting sizes. The alginate polymer in AELS is present as an interwoven network of fibres [18] with large aqueous inclusions and bead size reduction is likely to result from DMSO – water relationships impacting on the alginate polymer as the CPA permeates the internal aqueous spaces. Following cryopreservation and rewarming of the same cohort of AELS, in the thawed suspension, at chilled temperatures, before DMSO dilution, bead size remained reduced. During the 3 CPA washing steps using room temperature equilibrated diluents there was an incremental return of bead size towards control, starting size. Finally, on returning the AELS to culture medium at 37 C for 1 hr, bead sizes were not different from those of the control, starting cohort of beads.

Discussion

Exposure of AELS to DMSO, at a final concentration of 12% (v/v) for 10 minutes, in our standard CPA mixture for AELS. This also did not have a negative effect on the metabolic viability of the AELS 24 hours after dilution, irrespective of the exposure temperature between 37 C and 0 C. This confirms the empirical studies made in the development of our early cryopreservation regimes [14], that chilled solutions can be used safely. We know from previous work that these lower concentrations of DMSO were without effect on viability compared to batch-equivalent fresh controls when exposure times up to 40 min were investigated [18]. The results also concur with comments in a recent review on DMSO as a cryoprotectant which indicate that DMSO has negligible impact on cell viability as long as exposure times are kept as short as required to achieve efficacy for the permeating CPA [1]. Our current results give an indication that that transient exposure of the BAL biomass to DMSO, at temperatures even up to 37°C, during the thawing process does not negatively impact on the viability of the AELS. However, our data show that if DMSO exposure occurs at higher concentrations [40%] there was a clear toxicity signal even after only 10 minutes at 37 C. Nevertheless, from previous work, AELS can maintain good post-exposure viabilities after exposure to high DMSO concentrations as long as low temperatures are used for exposure [19]. We choose the conditions of high (40%) DMSO exposure to AELS at 37 C in our current studies and from previous experience [18] as conditions of high CPA toxicit to mimic what might happen if thawing stages were not carefully controlled for time before disappearance of the last ice crystals and starting the CPA dilution processes. As stated, in these atypical conditions, toxic effects of the DMSO were clearly seen. This adds weight, both, to the need to maintain rapid re-warming to ensure that the ice matrix dissolves quickly to avoid local areas of higher CPA concentrations in the partially-thawed mixture, and to the notion of sample agitation to mix any pockets of high localised CPA concentrations throughout the thawed aqueous solution, back towards the lower starting concentration. These comments are particularly relevant to cryopreservation in large volumes >1 litre where there are real possibilities of inhomogeneity in solution temperature and composition as the residual ice

finally disappears. Elmoazzen et al also investigated the concentration, time and temperature dependent toxicity of DMSO in articular cartilage and chondrocytes, finding significant toxicity at concentrations above 1M but limited toxicity at 1M; these authors suggested that DMSO at 1M may not be toxic in articular cartilage [4]

The thawing process itself can lead to injurious osmotic stress, as seen by Gupta et al in red blood cells [6], due to ice melting and liberation of osmotically active water in the extracellular space. As discussed by Pegg, cells can only tolerate small changes in cell volume without significant damage [17]. Therefore, it is important to minimise changes in cell volume during the CPA addition and removal steps. Osmotic injury could also impact the stability of the microbead units which carry the liver cell spheroids themselves. In our present study measurement of alginate bead diameter showed a gradual swelling of the bead as it was moved through solutions of changing osmolality, suggesting minimised osmotic injury. We know from previous work [15] that the alginate gel which we use in AELS is highly permeable to small molecules and even to polymers (e.g.150 kD dextran) so it will not impede DMSO diffusion . The osmotic responses of the microbeads themselves in our current study reflect the fact each bead is a network of polymerised alginate fibres, which presumably respond to the external osmotic changes by water movement out of the spherical body. One may consider that external osmotic changes may in some way alter the interactions between alginate fibres themselves, but the fact that microbead diameter quickly returned to pre-osmotic stress values argues against such a physico-chemical change in the polymerised alginate. Introducing the beads to a solution with a radically different osmolality can itself cause osmotic shock. Potentially, if the alginate scaffold and the entrapped liver spheroid were to swell at different rates, the differential expansion could cause detachment of the spheroid from the surrounding matrix, leading to detachment induced cell death [2].

Measurement of refractive index was effective in measuring the DMSO concentration on the post-thaw wash supernatant. We have applied this in previous preliminary studies [18]. The technique was also used in studies on organ perfusion with CPA by Wong et al [25]. Alternative methods of determining solute concentration in a solution such as, titration and absorbance measurement are unsuitable for DMSO as it does not appreciably change the pH or absorbance of a solution. Therefore, we were able to determine that the washing protocol removed all DMSO in the cryopreserved AELS, down to the limit of detection of the refractometer which is approximately 1% (w/v) (in the 1:2 & 1:3 bead:wash ratios). During the recovery phase from cryopreservation any residual DMSO will be diluted in 110L of culture medium (the total volume of media in the conditioning vessel) and during treatment the BAL biomass is part of loop with >10L plasma, further diluting the already undetectable concentration of DMSO remaining in the biomass. Therefore, patient contact with DMSO should not be a concern. However, for a full-size BAL (2.5L) this would be 7L of washing media per wash, whereas at a 1:2 ratio the volumes are more manageable.

Conclusions

Developing a cryopreservation recovery protocol suitable for GMP manufacture of the biological component of a BioArtificial Liver Machine is a key part of the logistical delivery to patients. Determining the removal of a potentially toxic component, DMSO, has indicated successful removal prior to exposure to patients, an important step in the translation pathway.

We have demonstrated that the thawing method minimised DMSO toxicity to the BAL biomass, enabling effective cryopreservation of the BAL for future clinical translation. The post-thaw washing protocol successfully removed all the DMSO present in the cryopreserved BAL, thereby minimising toxicity to the patient. The washing protocol has also been optimised to prevent osmotic injury to the biomass as it is gradually reintroduced to a physiological environment.

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Conflicts of Interest

Nothing to declare

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