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A liquidus tracking approach to the cryopreservation of human cartilage allografts ☆

A.G. Kay [a,b,d,*], J.A. Hoyland [c], P. Rooney [a], J.N. Kearney [a], D.E. Pegg [d]

[a] R&D Tissue Services, NHS Blood and Transplant, 14 Estuary Banks, Speke, Liverpool L24 8RB, UK
[b] Leopold Muller Arthritis Research Centre, Robert Jones and Agnes Hunt Orthopaedic Hospital Foundation Trust, Oswestry, Shropshire SY10 7AG, UK
[c] Centre For Tissue Injury and Repair, Institute of Inflammation and Repair, Faculty of Medical and Human Sciences, The University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK
[d] Biology Department, University of York, York YO10 5DD, UK

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ABSTRACT

In the "liquidus tracking" (LT) approach to cryopreservation both the temperature and the concentration of cryoprotectant (CPA) are controlled such that solution composition "tracks" the liquidus (melting point) line for that system. Ice crystal formation is prevented but the tissue is not exposed to CPA concentrations exceeding those experienced by cells during conventional cryopreservation. This approach is particularly appropriate for articular cartilage because chondrocytes *in situ* are exquisitely susceptible to damage by the crystallisation of ice. This project aimed to develop a suitable process for tissue to be used in the surgical repair of damaged human knee joints. A high proportion of the chondrocytes should be alive.

Human articular cartilage was obtained from deceased donors and dimethyl sulphoxide (DMSO) was used as the CPA, cooling was at 0.14 °C/min and warming at 0.42 °C/min. The vehicle solution was CPTes2. A program of increasing DMSO concentration was developed for cooling and this gave satisfactory tissue concentrations but reduction of DMSO concentration during warming was inadequate, resulting in higher tissue concentrations than required. Biomechanical testing indicated a compressive modulus of 9.5 ± 1.3 MPa in LT-processed cartilage, with control values of 11.6 ± 0.8 MPa ($p > 0.05$, Student's *t*-test). Measurement of GAG synthesis sometimes approached 65% or 85% of control, but the variability of replicate data prevented firm conclusions. Ideally allograft tissue should score 1A or above on the Noyes scale and the donor age should be less than 46 years but the cartilage used in this study did not meet these standards.

Introduction

The basic idea of the "liquidus tracking" (LT) approach to cryopreservation is to control the concentration of CPA as well as temperature such that the solution composition "tracks" the liquidus (melting point) line for that system [18]. If tracking is precise, the crystallisation of ice is prevented but the tissue is not exposed to CPA concentrations exceeding those experienced during conventional cryopreservation. The idea was originated by Farrant [9], exploited by Elford in 1972 [8] and then applied to cartilage by Pegg et al. in 2006 [20] and Wang et al. in 2007 [25]. The aim of this study was to discover whether the LT approach could be applied to the cryopreservation of human articular cartilage. Cryopreserving cartilage by standard methods is difficult due to the exquisite susceptibility of chondrocytes *in situ* to damage by the crystallisation of ice in the chondrons [21].

The diffusion process that is used to replace tissue water by cryoprotectant (CPA) requires time and is retarded by cooling. In fact, the intracellular concentration of a penetrating CPA (we have chosen dimethyl sulphoxide – DMSO) is achieved partly by the

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* Corresponding author at: Leopold Muller Arthritis Research Centre, Robert Jones and Agnes Hunt Orthopaedic Hospital Foundation Trust, Oswestry, Shropshire SY10 7AG, UK.
E-mail address: A.G.Kay@keele.ac.uk (A.G. Kay).

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osmotic extraction of water because water diffuses more rapidly than DMSO. The important consideration is the concentration of DMSO that is achieved in the aqueous compartment of the tissue which can be measured to ensure that liquidus conditions have been achieved. The eutectic temperature of the system DMSO/NaCl/water is ~−70 °C for compositions used here [18]. Below this temperature vitrification occurs and a glass is formed.

The conventional approach to cryopreservation by vitrification is to expose the tissue to a very high concentration of cryoprotectant and then to use rapid cooling and even more rapid warming to avoid the formation of ice. For example, Brockbank et al. [5] used 83% CPA and cooling at 43 °C/min, with warming at 225 °C/min for discs of porcine cartilage. Jomha et al. [11] used 47.4 w/w CPA, unquantified rapid cooling and warming at 64–122 °C/min with human osteochondral dowels. This approach involves exposure to very high concentrations of CPA at relatively high temperatures, thereby risking enhanced toxicity, and because the maximum tolerated concentration of CPA is insufficient to prevent the formation of ice under equilibrium conditions, high rates of cooling and warming are required to avoid freezing. LT theory avoids both problems: the solute conditions are the same as those in conventional cryopreservation but no ice is formed. This paper describes the experimental development of a GMP- compliant LT system for human knee joint condyles.

Preliminary work on automated LT with continuous stirring was published by Wang et al. [25] and was tested using osteochondral plugs and discs of ovine and human cartilage. A computer-controlled system was used to mix high and low concentration DMSO in a vehicle solution (CPTes2) [24] to achieve the desired DMSO concentration during program-controlled cooling. A 2 ml sample chamber with a flow rate of 1 ml/min was used. We refer to this approach as the "flow-through" method of LT. We have explored flow-through systems for human osteochondral dowels 2 cm in diameter but consultation with orthopaedic surgeons indicated a preference for larger pieces of cartilage that could be cut to appropriate size and shape in the operating room. The volume of solution required for the flow-through method, however, exceeded 15 L – which we considered unworkable. We then considered two other approaches to the process. The first used a single sample chamber to which sufficient DMSO was gradually added, under computer control, to produce the required solution composition while allowing the solution volume in the chamber to increase. However, the total volume of the sample holder was >10 L – again impractical. Finally, we designed a DMSO addition and removal method where the solution volume in the chamber was kept constant: this required a total solution volume of approximately 1 L during cooling and 2 L during warming. This was considered to be more practical and in this paper we describe the development of a computer-controlled LT system using the constant volume approach for human articular allografts. The specific objective was to develop an LT preservation process for articular cartilage allografts suitable for the surgical repair of lesions in the knee joint.

The presence of living chondrocytes has been directly correlated with an improved outcome following surgical procedures [4]. Less than 20% of chondrocytes in conventionally cryopreserved cartilage survive and hypothermic storage, the method currently in use, shows a marked decline in cell function and metabolism following 14 days storage at 4 °C [2,16,27]. It was considered important that a high proportion of the chondrocytes, say a minimum of 70% should remain alive. The assay of functional survival used in this study was the ability of the processed tissue to synthesize glycosaminoglycans (GAGS). The theoretical advantage of living cartilage grafts is that the chondrocytes should then survive in the graft and continue to contribute to sustaining the surrounding matrix [10,14]. In this connection it is important to recognize that the practice of quoting the results of metabolic assays or membrane integrity staining as a measure of cell "viability" is open to challenge. When high "viability" scores have been reported, this has not necessarily been reflected in good functional results: for example, following hypothermic storage at 4 °C for 14 days the "Live/Dead" BCECF-AM assay gave 98.3% recovery whereas the ^{35}S uptake was 39.2% [26]. In other studies the same assay gave 82 ± 5%, whereas ^{35}S uptake was 64 ± 19% [2] or 55.0 ± 8.5% when the ^{35}S uptake was 34.3 ± 6.7% [16]. These data reinforce the importance of selecting a rigorous assay when assessing the quality of preserved grafts.

Methods

Tissue samples

Human articular cartilage was obtained from deceased donors within 48 h of death. Consent to utilize knee tissues was obtained in all cases, and cartilage was retrieved at facilities that held a Human Tissue Authority pathology license permitting removal of tissues for non-therapeutic use. A total of 42 donations were used in this work: 32 male and 10 female. The age range overall was 25–79 years, the mean age of male donors being 55.2 ± 13.4 years (mean ± SD) and of female donors, 55.8 ± 14.6 years. The average post-mortem processing time was 5.8 ± 3.3 days ranging from 2 to 12 days post-mortem. The tissue was stored in DMEM:F12 (Sigma–Aldrich) at 4–8 °C until use. All samples that were used gave acceptable baseline results in the GAG functional assay (within the range 6–59 CPM/mg wet weight of tissue, approx. 13–413 CPM/mg when adjusted to dry weight). A single hemi-condyle from each donor was used. Only tissues without significant lesions or degradation in cartilage quality were used. Tissue was removed from the distal femur providing a piece approximately 5 cm deep and 10 cm across at the widest point. Within 24 h excess bone and soft tissue was removed and the condyles were bisected into medial and lateral components using a bone/tissue saw (deSoutter), removing the central region of the trochlear groove. The tibial plateau was not used in this study. Where comparisons with controls were made, the control tissue was sampled and tested at the same time as the processed material.

Preparation of solutions

The vehicle solutions used included the standard chondrocyte culture medium DMEM:F12 (1:1) and CPTes2, a TES-buffered solution designed for corneal preservation [24]. Both solutions were made up on a w/w basis with DMSO treated as part of the solvent. All compositions were calculated to be isotonic in the presence of CPA as described by Pegg [17]. To achieve this, the components required for 1 L of normal strength solution were dissolved in an appropriate mass of water such that water + DMSO = 1000 g. In all cases the initial exposure of cartilage to CPA was to 20% w/w DMSO at 0 °C in CPTes2 [12].

Assay of DMSO concentration in tissue

The DMSO concentration in tissue was determined by measurement of water by a Karl Fischer back-titration method and of DMSO by an HPLC method as previously described [20–22] The tissue DMSO concentration was calculated using:

$$\frac{d \times 100}{w + d}\ \%w/w.$$

where:

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