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Ethylene glycol and glycerol loading and unloading in porcine meniscal tissue

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ABSTRACT

The development of a long-term storage method for meniscus, a complex tissue of the knee prone to injury, would improve the procedure and outcomes of meniscus transplantation. Cryopreservation uses cryoprotective agents (CPAs) including ethylene glycol (EG) and glycerol to preserve a variety of live tissues, and understanding of the CPA permeation kinetics will be critical in designing a vitrification protocol for meniscus.

The purpose of this preliminary study was to understand the loading and unloading behaviours of EG and glycerol in meniscus by observing their efflux. For the main experiment, lateral and medial porcine menisci were incubated with CPA for 24 h at three temperatures (i.e., 4, 22, and 37 °C). Then, the menisci were immersed in 25 ml of X-VIVO™10 and CPA efflux was recorded by monitoring the molality of two consecutive washout solutions at different time points. In a subsequent experiment, menisci were incubated in the CPA solutions for 48 h at 22 °C, and the results were compared to those obtained at 22 °C in the main experiment.

Results showed a rapid efflux of CPA from meniscus at the beginning of each wash. With increasing temperature, the amount of CPA efflux (and hence loading) increased. Using 24 h incubation, EG loaded the menisci more completely than glycerol. But after 48 h of incubation, both EG and glycerol achieved approximately the same degree of meniscus loading.

This study provides preliminary data that will facilitate future design of experiments aimed at development of meniscus permeation studies.

1. Introduction

The prevalence of meniscal injuries has led to the development of various approaches to alleviate patients' symptoms [17]. Arthroscopic meniscal repair and removal of either all or damaged portions of the meniscus (total or partial meniscectomy) are two of the most commonly employed surgical approaches in meniscal injuries [9,10]. Unfortunately, both approaches suffer from anatomical and biomechanical limitations, rendering them incomplete solutions to the meniscal injury problem. For instance, arthroscopic repair is limited to the vascularized, outer, 10–30% portion of the meniscus [7,34] and arthroscopic meniscectomy creates an area inside the knee joint that is devoid from protective meniscal tissue [6,12], resulting in poor long term outcomes with degenerative joint lesions [6,15,28,50] that will advance to osteoarthritis. However, as the presence of an intact meniscus is essential for normal function of the knee joint [33], meniscal allograft transplantation is an effective alternative that can restore normal knee anatomy and biomechanics in patients with non-salvageable meniscal injury [13,14,42,47,52]. Success of meniscal allograft transplantation depends on proper size and shape matching of the transplanted meniscus to the native meniscus [35,46], as well as the presence of viable cells to maintain the biosynthetic activity and biomechanical properties of the tissue [54]. In addition, the procurement of infection-free allograft [11,36] is another essential

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factor to ensure the recipient's safety. Freshly harvested meniscal allografts provide the highest number of viable cells [23] and, for best results, tissue recovery should be done within 4–6 h after donor death [23]. However, fresh allografts can maintain live cells for only up to 5 days at above 4 °C [40,41] thereby demanding rapid access for surgical transplantation. This short time frame, along with the need for size matching and infectious disease testing limits the clinical application of fresh meniscal grafts.

The availability of a long-term storage method for meniscal tissue would allow donor menisci to be stored in a tissue bank, leading to an increased number of available grafts, and would provide the necessary time for size matching, infectious disease screening and surgical team coordination [51,53]. Commonly used meniscal preservation techniques such as deep freezing and conventional cryopreservation have been found to result in less than optimal survival and biochemical changes in human and animal menisci [8,18,21,23,31,40,43]. Gelber et al. [21], using transmission electron microscopy, reported thinning and disruption of collagen fibrils in 13 human menisci after deep-freezing to −80 °C for 7 days without using cryoprotective agents (CPAs). Likewise, in the absence of CPAs, Reckers et al. [40] reported progressive loss of cellularity and structural damage in 180 rabbit menisci after freezing to three different subzero temperatures (−7.2 °C, −21.4 °C and −73 °C) [40]. Moreover, another study has indicated complete loss of cellularity and tissue destruction after only 2 weeks of deep freezing [18].

Cryopreservation, due to the use of cryoprotective agents (CPAs) and a slow freezing rate to inhibit ice crystal formation in cells, is expected to be less damaging than deep freezing. Several studies investigated the effect of cryopreservation on meniscal tissue; all were carried out using protocols adopted from articular cartilage studies and showed reduced cell viability with structural deterioration [8,18,23]. Fabbriciani et al. [18] studied the post-transplantation effect of a standard cryopreservation using 10% v/v dimethyl sulfoxide (Me_2SO) at 4 °C (following an articular cartilage based technique) in a 15 Tibetan goat menisci transplantation model. The authors reported normal appearance of menisci at different examination periods with best results observed at 2 weeks post-transplantation [18]. However, after the second week cell number started to decrease with increased water content and progressive decreases in the glycosaminoglycan (GAG) content over time. Furthermore, there was no difference when compared to deep frozen grafts [18]. Jackson et al. [23] cryopreserved 10 goat medial menisci that were incubated at 4 °C for 60 min in a solution containing 3% v/v glycerol and 3% v/v Me_2SO, followed by controlled rate freezing and storage in vapor phase of liquid nitrogen (−156 °C) for 30 days. The authors reported a post-thawing cell viability of 30% with increased water content and reduced GAG content when compared to control menisci [23]. Arnoczky et al. [8] examined five dog menisci that were incubated in a 4% v/v Me_2SO solution for 120 min at room temperature followed by controlled rate freezing to −100 °C and storage in liquid nitrogen (−196 °C) for 7 days. The authors reported no effect in the morphological appearance of menisci but there were alterations in the mechanical properties with only 10% of the cells remaining metabolically active [8]. These findings indicate the need for a more effective preservation technique that will not jeopardize the structural integrity and cell viability of the meniscus.

Vitrification or ice-free cryopreservation offers a promising means of preservation [22,25] by eliminating ice formation to preserve the tissue's structural integrity with improved levels of cellular viability [20]. Successful vitrification requires high concentrations of CPAs to keep the tissue super-cooled below −130 °C in an amorphous glassy state [25,38,39], creating a suspended animation in which tissue can be stored indefinitely. Typically, CPAs are added and removed in a stepwise fashion to avoid the osmotic damage that can result from single step addition or removal. Determination of appropriate tissue incubation times and temperatures should be based on understanding permeation kinetics of the desired CPAs upon which future studies can be built [3,24,26,45]. For the meniscus, there is no literature that provides basic understanding of the CPA behavior or distribution in this complex tissue. Furthermore, as was mentioned earlier, most reports that attempted to cryopreserve the meniscus used methods that were adopted from classical cryopreservation of cells in suspension as applied to articular cartilage with only limited success. There is one study [32] in the literature that examined mechanical properties in pig menisci that were vitrified in Me_2SO using techniques that were adopted from studies performed on different tissues. The actual vitrification protocol was not described in that study and the experiment focused only on the mechanical properties of the menisci and not cell recovery. Li et al. [32] reported that vitrified minipig menisci displayed mechanical properties that were slightly lower but comparable with normal menisci.

Successful vitrification of complex, organized tissue is difficult [19,38] and a comprehensive approach towards tissue vitrification starts with understanding the permeation kinetics of certain CPAs into the target tissue [1,4,26,30,37]. Permeation kinetics can be accurately predicted from applying mathematical modeling to experimental findings, including surface dimensions, in order to describe the mass transport of CPA through the extracellular matrix [2,4], but first an experimental understanding of the mass transport in a particular tissue is needed. Our laboratory has published a report of successful vitrification of intact human articular cartilage (a tissue that has been very difficult to cryopreserve) with high cell viability, metabolic activity and function [25,27]. This breakthrough was preceded by a series of extensive experimental and mathematical investigations to understand the CPA permeation behaviors [2–4,26,29,45,55] and supported with later efflux studies [44,55]. There are structural and functional similarities between articular cartilage and the meniscus that indicate that we can follow our articular cartilage strategy to develop future strategies for meniscus. However, unlike articular cartilage, the meniscus is a geometrically complex three-dimensional tissue that is partially vascularized and so further understanding is needed about transport of CPAs in this tissue before modeling can begin. We recently performed an anatomical study that provided detailed dimensional measurements for the body of the human, sheep and pig meniscus [48]. Sheep and pig meniscus are anatomically similar to human meniscus and we chose the pig because it is a common animal model for meniscal study and is slightly larger than human meniscus. Thus, we thought it would be a good model for developing cryoprotectant transport understanding that will lead to conservative design. We undertook this study as a first step towards formulating a basic understanding of the loading and unloading behaviors of EG and glycerol (two common CPAs used in articular cartilage cryopreservation) in medial and lateral porcine menisci. This understanding is expected to help in designing future studies aimed at describing the permeation kinetics of the chosen CPA with the meniscus. In this study, we measured the efflux of two CPAs out of pig menisci at various temperatures.

2. Materials and methods

2.1. Solutions and materials

Pure CPAs were purchased from Fisher Scientific (Fair Lawn, NJ, USA). CPAs used were ethylene glycol (Certified) and glycerol (Spectranalyzed®). X-VIVO™10, a serum-free medium (Lonza Walkersville, MD, USA) was used to prepare all CPA solutions in the

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