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Effects of freezing-induced cell–fluid–matrix interactions on the cells and extracellular matrix of engineered tissues

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ABSTRACT

The two most significant challenges for successful cryopreservation of engineered tissues (ETs) are preserving tissue functionality and controlling highly tissue-type dependent preservation outcomes. In order to address these challenges, freezing-induced cell–fluid–matrix interactions should be understood, which determine the post-thaw cell viability and extracellular matrix (ECM) microstructure. However, the current understanding of this tissue-level biophysical interaction is still limited. In this study, freezing-induced cell–fluid–matrix interactions and their impact on the cells and ECM microstructure of ETs were investigated using dermal equivalents as a model ET. The dermal equivalents were constructed by seeding human dermal fibroblasts in type I collagen matrices with varying cell seeding density and collagen concentration. While these dermal equivalents underwent an identical freeze/thaw condition, their spatiotemporal deformation during freezing, post-thaw ECM microstructure, and cellular level cryoresponse were characterized. The results showed that the extent and characteristics of freezing-induced deformation were significantly different among the experimental groups, and the ETs with denser ECM microstructure experienced a larger deformation. The magnitude of the deformation was well correlated to the post-thaw ECM structure, suggesting that the freezing-induced deformation is a good indicator of post-thaw ECM structure. A significant difference in the extent of cellular injury was also noted among the experimental groups, and it depended on the extent of freezing-induced deformation of the ETs and the initial cytoskeleton organization. These results suggest that the cells have been subjected to mechanical insult due to the freezing-induced deformation as well as thermal insult. These findings provide insight on tissue-type dependent cryopreservation outcomes, and can help to design and modify cryopreservation protocols for new types of tissues from a pre-developed cryopreservation protocol.

1. Introduction

Successful cryopreservation of a wide variety of biomaterials is critically important to cell/tissue engineering and regenerative medicine because it can provide long-term storage and "off-the-shelf" availability of various cell/tissue engineering products [1–3]. Although successful preservation of cellular and/or simple tissue systems in the frozen state have been reported [4–6], it is still challenging to design and develop cryopreservation protocols for a wide variety of engineered tissues (ETs). The two most significant challenges are – 1) preserving tissue functionality, and 2) controlling highly tissue-type dependent preservation outcomes. Tissue functionality includes mechanical, optical and transport properties of ETs, which are critical to the physiological functions of ETs. Due to the wide spectrum and range of the functional properties of ETs, it is extremely difficult to develop a cryopreservation protocol targeting any given properties. Tissue-type dependent cryopreservation outcomes indicate that a successful cryopreservation protocol for any given type of tissue is difficult to adapt to others types of tissues [7,8]. These two challenges are caused by lack of mechanistic understanding of tissue-level response to freezing and its effects on tissue functionality, and they become

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more significant as more diverse cell/tissue engineering products are developed.

Since many of the tissue functional properties are associated with the microstructure of the extracellular matrix (ECM), preserving the microstructure and integrity of the ECM may be the key for successful cryopreservation of functional ETs. Besides providing a structural scaffold and determining the functional properties, the ECM plays significant roles in tissue physiology through interaction with cells and interstitial fluid transport. These roles include regulating cell morphology and growth [9,10], and intercellular signaling [11]. The ECM can also be reconfigured by cells during tissue remodeling and wound healing [12–14]. Thus, in order to cryopreserve functional tissue, its ECM microstructure should be maintained during cryopreservation as well as the cellular viability [15–17].

In spite of its significance, the effects of freeze/thaw (F/T) on the post-thaw ECM microstructure are not well understood. During cryopreservation, ice is formed in both the intra- and extracellular spaces, and extracellular ice formation (EIF) is thought to induce damage to the ECM microstructure. However, the extent of EIF and subsequent damage to the ECM microstructure are not well understood. Several studies have been performed to investigate the morphology of EIF, but its impact on the post-thaw ECM structure was not fully studied [18–21]. Later, the matrix of post-thaw cartilage was characterized using MRI, and significant MRI signal changes were observed [22]. A multiphoton-induced autofluorescence and second harmonic generation microscopy were performed on cryopreserved and vitrified cartilage, and these also reported changes to the post-thaw collagenous matrix structure [23]. Both studies recognized the F/T-induced changes to the ECM microstructure, but their measurements did not provide quantitative information on the underlying biophysical mechanisms during F/T. On the contrary, retained structural integrity of collagen and elastin within cryopreserved human heart valve tissue was reported using two-photon laser scanning confocal microscopy [15]. More recently, the collagen structure of fresh and cryopreserved arteries was compared using second harmonic generation microscopy [24]. However, due to limited understanding of tissue-level biophysical phenomena during F/T, no mechanistic strategy has been established yet as to how to preserve the ECM microstructure as well as the cellular viability.

A few recent studies proposed that freezing-induced spatiotemporal deformation of the tissue determines the post-thaw ECM microstructure and ultimately the functionality of ETs [25,26]. Han et al. [25] performed a theoretical study based on a poroelastic model and suggested that freezing of tissue may induce the spatial and temporal redistribution of interstitial fluid and subsequent spatiotemporal ECM swelling. In addition, the post-thaw microstructure of the collagen matrices was visualized to support the theoretical results. In a later study by Teo et al. [26], the spatiotemporal deformation of ETs during freezing was measured using a quantum dot mediated cell image deformetry technique. The results confirm that freezing-induced complex deformation patterns of the ET as the freezing interface propagates, which might result from the interactions among the cells, ECM and interstitial fluid. Thus, understanding of the freezing-induced cell–fluid–matrix interactions could enable the mechanistic prediction of the post-thaw ECM microstructure and cell viability of various ETs. Moreover, this could also lay the groundwork for the knowledge-base to design and modify cryopreservation protocols for various types of ETs.

The objectives of this study, thus, are to delineate the role of each component of the cell–fluid–matrix interactions during freezing, and to characterize the effects of these interactions on the cells and ECM of ETs. To achieve these objectives, we designed experiments with dermal equivalents, which are constructed by seeding human dermal fibroblasts in type I collagen matrices, with different microstructures by varying cell seeding density and collagen concentration. Three different experimental groups were prepared and used: the nominal group (NORM), the high cell density (HCEL) group, and the high collagen concentration (HCOL) group. While these dermal equivalents underwent an identical F/T protocol, their spatiotemporal deformation during freezing was measured using the cell image deformetry technique. After F/T, the ECM microstructure was characterized using the scanning electron microscopy technique. The freezing-induced deformation and post-thaw ECM structural changes of all experimental groups were cross-compared and analyzed to delineate the role of each component in the cell–fluid–matrix interactions during freezing. The cryoresponse of fibroblasts was characterized by assessing the post-thaw cell viability, cell–matrix binding and cytoskeleton organization. These results were also correlated to the freezing-induced deformation of ETs and further discussed considering the implications for cryopreservation of functional ETs. The implications for cryopreservation of various types of tissues are also discussed in the context of addressing tissue-type dependent cryopreservation outcomes.

2. Materials and methods

2.1. Cells and reagents

Early-passage hTERT-immortalized human dermal fibroblasts [27] were maintained in culture medium (DMEM/F12, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin. The fibroblasts were cultured up to the 15th passage in 75 cm^2 T-flasks at 37 °C and 5% CO_2. The cells were consistently harvested at 80% confluency by using 0.05% trypsin and 0.53 mM EDTA.

2.2. Engineered tissues with quantum dot-labeled fibroblasts

ETs (i.e., dermal equivalents) for use in freezing-induced tissue deformation measurements were prepared by seeding quantum dot (QD)-labeled fibroblasts in collagen matrices. The detailed preparation procedure has been described elsewhere [26]. Briefly, the collected cells were first labeled with QDs (Qtracker 655, Invitrogen, Carlsbad, CA) according to the protocol suggested by the manufacturer (the cellular uptake of QDs was characterized and is shown in Figure S1). After labeling, the cells were suspended in 2 mL of collagen solution, which was prepared from high concentration type I rat tail collagen (BD Biosciences, Bedford, MA). The collagen solution with the cells was placed in a 48 × 18 mm chamber slide (Lab-Tek II, Nunc, Naperville, IL) and allowed to polymerize at 37 °C for 60 min. After polymerization, 2 mL of complete medium was added, and the ET was incubated for 24 h before the freezing experiments. Three different experimental groups were prepared by varying cell seeding density and collagen concentration: the nominal (NORM) group (cell seeding density = 2×10^5 cells/mL and collagen concentration = 3 mg/mL), the high cell density (HCEL) group (cell seeding density = 4×10^5 cells/mL and collagen concentration = 3 mg/mL), and the high collagen concentration (HCOL) group (cell seeding density = 2×10^5 cells/mL and collagen concentration = 6 mg/mL). The cell seeding density and collagen concentration of the NORM group were determined based on the values typically used for constructing dermal equivalents [28–31], and two-fold increased cell density and collagen concentration were used for the HCEL and the HCOL groups, respectively.

2.3. Cell image deformetry

Cell image deformetry (CID) was used to measure the spatiotemporal deformation of ETs during freezing. A detailed description of the CID technique can be found elsewhere [26]. Briefly, the ET was frozen on a directional solidification stage (Figure S2A). The local temperature history of the ET was characterized and is presented in Figure S2B. A fluorescence macro/microscope (MVX10, Olympus, Center Valley, PA) equipped with a long working distance objective lens and a TRITC filter was used to visualize the QD-labeled cells of the ET during freezing. The ET was continuously imaged with a 1 s interval using a high sensitivity CCD camera (PIXIS 512, Princeton Instruments, Trenton, NJ). The acquired sequential images were cross-correlated at a 10 s interval using commercial software (DaVis 7.1, LaVision, Ypsilanti, MI) to determine the local deformation rates (μm/s). These deformation rates were further analyzed to estimate dilatation. Dilatation (s^{-1}) was defined as the rate of area increase per unit area, i.e., expansion, as follows: