Advancing Fresh, Viable Meniscal Allograft Preservation: Ice-free Vitrification in a Pig Model

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INTRODUCTION: The successful transplantation of musculoskeletal tissues plays a pivotal role in orthopedic and reconstructive surgeries. However, the scarcity of fresh allografts poses significant obstacles to their widespread clinical implementation. This challenge is particularly evident in the case of meniscal allograft transplantation (MAT). While MAT has demonstrated promising long-term outcomes, its potential is hindered by the limited number of procedures performed. Despite the substantial demand for meniscus replacement among hundreds of thousands of patients, only around 2000 surgeries are conducted annually in the United States. Efforts have been made to address the shortage of fresh allografts through preservation methods such as fresh-freezing at -80°C and conventional cryopreservation using slow-freezing at -196°C. However, these approaches have proven to be less than optimal, as they lead to cellular loss and tissue damage caused by the formation of ice crystals during the freezing process. This compromises the outcomes following transplantation. An alternative strategy involves vitrification, which preserves living cells without ice formation. However, applying vitrification to larger musculoskeletal tissues like menisci (>3 mL) presents a significant challenge. This is primarily due to the complex, dense, and avascular structure of the meniscus, which makes it difficult to effectively regulate the loading periods of cryoprotectants (CPAs). Achieving adequate penetration of CPAs while minimizing their toxic effects becomes a formidable task. In this study, a simulation-based optimization approach combining computational modeling with non-invasive and non-destructive microcomputed tomography (μCT) imaging was developed to address this challenge. This approach accurately predicts three-dimensional CPA concentrations and distribution throughout the entire meniscus, enabling successful preservation of viable menisci at cryogenic temperatures through vitrification. This advancement holds the potential to revolutionize the field of MAT by overcoming the limitations posed by traditional freezing methods and providing a more effective way to preserve and transplant meniscal tissues.

METHODS: For the proof of concept, meniscal specimens with two thicknesses (1 mm and 3.5 mm thickness) were utilized. To assess viability, live/dead staining and alamarBlue assays were performed on fresh, slow-frozen, and vitrified samples. Computational modeling, coupled with μCT imaging, facilitated the determination of CPA diffusivities, thus enabling prediction of CPA loading protocols for vitrification of whole meniscus. According to the simulations, subsequent vitrification of complete menisci employed loading periods of 2 hours and 3 hours for CPAs. Following convective warming of vitrified whole menisci, three distinct regions encompassing the anterior horn, the central region, and the posterior horn underwent examination at both cellular and tissue levels. Viability assessments were carried out on transverse and sagittal sections via fluorescence live-dead staining and alamarBlue assays. Hematoxylin and eosin (H&E), Safranin O, and Sirus red staining were employed for histological analysis of the extracellular matrix (ECM) structure and compositions. Microindentation testing was employed to determine the biomechanical properties of the tissues.

RESULTS SECTION: For the 1 mm thick tissues (4 mL), the vitrified samples exhibited preserved living cells comparable to fresh samples, whereas the slow-frozen tissues showed a substantial reduction in cell viability. However, with an increase in thickness to 3.5 mm (4 mL), both vitrification and slow freezing resulted in a decline in cell viability. Nonetheless, when the loading time was extended to 3 hours, the vitrified 3.5 mm tissues maintained living cells across their entire thickness. These findings suggest that viability of vitrified samples highly depends on the concentrations of diffused CPAs (**Fig 1**). CPA diffusivities were quantified and used to predict concentration profiles throughout the whole meniscus (**Fig 2**). The lowest CPA concentrations in each region exhibited a penetration of 50-75% VS55 through the entire meniscus following a 3-hour loading period and therefore the 3-hour loading protocol was used for vitrification of whole meniscus (10 mL). As expected, the 3-hour vitrification showed a significantly higher viability in whole menisci, achieving an average of approximately 70%, compared to slow freezing and 2-hour vitrification groups (**Fig 3**). Furthermore, minimal alterations were observed in the extracellular matrix (ECM) structure and biomechanical strength of vitrified menisci following the 3-hour CPA loading process, compared to untreated fresh tissues.

DISCUSSION: These findings offer compelling evidence that 1) the critical influence of diffused CPA concentration on the viability of vitrified larger-sized meniscal tissues; 2) Our newly developed simulation-based optimization approach effectively controls the loading periods of CPAs; 3) Through the utilization of the optimized CPA loading protocol, we successfully scaled up the volume to 10 mL and achieved the presentation of large, viable whole menisci through vitrification, making a significant improvement over the conventional slow-freezing method. 4) This pioneering technology establishes a clear path toward enabling the vitrification of human meniscal allografts and even larger-sized musculoskeletal allografts.

SIGNIFICANCE/CLINICAL RELEVANCE: This advanced preservation technology has the potential to alleviate the shortage of viable human meniscal allografts in MAT, improving post-meniscectomy knee treatments by maintaining cell viability and tissue integrity under cryogenic conditions.

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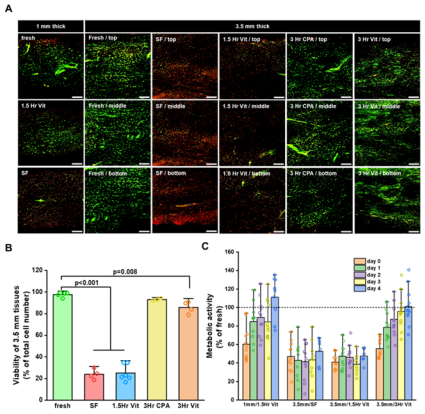

Fig 1. (A) Live/dead images of small meniscal specimens; (B) quantitative analysis of live/dead images; (C) metabolic activity measured by the alamarBlue assay.

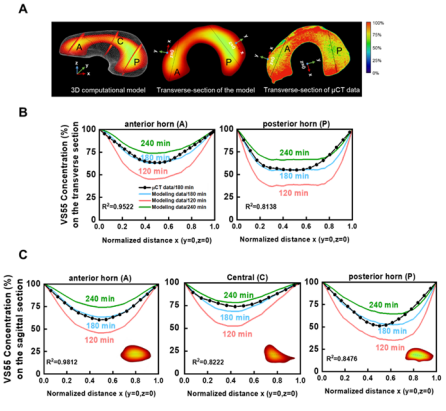

Fig 2. (A) Simulation and validation of CPA concentrations in the entire meniscus; (B) concentration profiles on transverse section; (C) concentration profiles on sagittal sections.

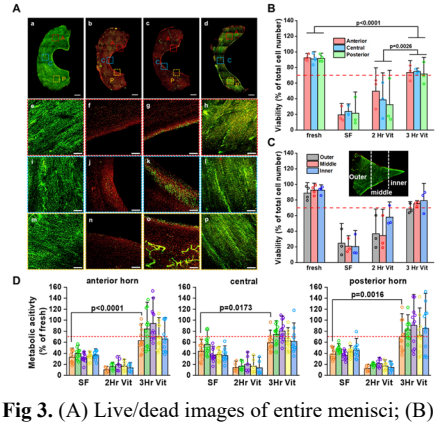

Fig 3. (A) Live/dead images of entire menisci; (B) quantitative analysis of live/dead images in different regions; (C) quantitative analysis of live/dead images in different layers; (D) metabolic activity measured by the alamarBlue assay.