Impact of Hypothermia upon Chondrocyte Viability and Cartilage Matrix Permeability after 1 Month of Refrigerated Storage

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Preservation · Hypothermia · Metabolism · Cartilage · Chondrocyte · Matrix · Storage · Permeability · Viability

Summary
Background: The purpose of this research was to assess the extracellular matrix and chondrocytes of articular cartilage during refrigerated storage and to determine whether changes could be detected in the time frame that cartilage is stored for clinical use. Methods: Porcine cartilage was stored as either bisected femoral heads with bone attached or plugs without the underlying bone in culture medium with fetal bovine serum for 1 month at 4 °C. Metabolic activity was tested using a resazurin reduction method on intact tissue and viable cell recovery after enzymatic tissue digestion at each time point. Cartilage plug permeability was evaluated by measuring electrical conductivity. Results: Storage in culture medium provided good cartilage viability and metabolic function for 7 days; however, significant changes were observed in femoral heads (p < 0.05). All mean chondrocyte assessment values were <30% of fresh controls at 28 days. Cartilage plugs tended to perform better after 7 days of storage than the femoral heads and retained significantly higher metabolic activity (mean = 94.5% vs. 70.5%; p < 0.05). Cartilage plugs demonstrated consistent changes in electrical conductivity after 28 days of storage (p < 0.05). Conclusion: Refrigerated storage of cartilage results in both loss of chondrocyte viability and matrix permeability.

Schlüsselwörter
Biologische Konservierung · Hypothermie · Stoffwechsel · Knorpel · Chondrozyten · Matrix · Lagerung · Permeabilität · Viabilität

Zusammenfassung
Introduction

Fresh donor-derived osteochondral plugs and intact osteochondral grafts harvested within 24 h of donor death and banked at 4 °C for up to 42 days are employed clinically for repair of cartilage defects. Plugs are employed in mosaicplasty procedures for smaller defects less than 3 cm wide and less than 1 cm deep. Osteochondral allograft transplantation has been an effective treatment option with promising long-term clinical outcomes for larger focal posttraumatic defects in the knee for young, active individuals [1]. There were 15,797 allogeneic human articular cartilage procedures performed in the USA during 2006 [2].

Review of the literature generally advocates articular cartilage storage for 2–6 weeks. However, in our experience cells typically survive less than 48 h of cold storage in culture media without specially designed hypothermic storage solutions [3]. There are exceptions, such as myofibroblasts in heart valve leaflets [4] or chondrocytes in articular cartilage. There have been reports on chondrocytes in articular cartilage of humans [5–9] and several animal species including rabbits [10], rats [11, 12], dogs [13, 14], miniature pigs [15], sheep [16], cattle [17, 18], and non-human primates [9], demonstrating that chondrocytes survive days or weeks of hypothermic storage in their natural extracellular matrix. The take-home message from these studies is that chondrocyte viability decreases with time in storage and that the rate of decline is influenced by the storage solution employed. However, many of these studies used fluorescent assays to determine the impact of storage, and doubt has been cast upon the accuracy of such assays for chondrocytes in intact cartilage [19]. Another concern, if most of the cells are viable, is the possibility that prolonged storage of cartilage with viable cells might promote the release of enzymes such as metalloproteinases (MMPs), which may impact the permeability of the extracellular matrix (ECM).

Therefore, we initiated research to assess the impact of 4 °C storage on cartilage cell viability and ECM permeability during storage in tissue culture medium. In this baseline study we employed porcine cartilage comparing large pieces of cartilage (bisected femoral heads) versus cartilage plugs to determine whether or not tissue volume had an impact on chondrocyte viability using two assays. We also assessed cartilage plug permeability by measuring electrical conductivity.

Material and Methods

No animals were sacrificed for these studies. Bona fide excess tissue was employed. Bona fide excess tissue is a term used to describe animal-derived materials obtained from animals after they have been sacrificed for other uses. Pig knees were procured from adult domestic Yorkshire cross farm pigs (25–60 kg) at the conclusion of other Institutional Animal Care and Use Committee-approved research projects at the Medical University of South Carolina. The knees were placed in zip lock bags with an iodine solution and transported on ice to our laboratory for aseptic dissection.

Experimental Design

Porcine femoral cartilage, either bisected femoral heads consisting of both cartilage and bone or cartilage plugs without the underlying bone, were stored in culture medium (DMEM) with 10% fetal bovine serum (FBS) for 0 (fresh untreated control), 1, 7, or 28 days at 4 °C. The medium was changed weekly.

Viability Assessment

Chondrocyte metabolic activity was assessed using the resazurin reduction method after storage of the 6-mm discs for 0, 7, or 28 days. The resazurin reduction assay incorporates a water-soluble fluorometric viability oxidation reduction (REDOX) indicator which detects metabolic activity by both fluorescing and changing color in response to chemical reduction of the growth medium. Metabolically active cells reduce resazurin to fluorescing resorufin [20]. Tissue samples were placed in 37 °C culture conditions for 1 h to permit adjustment to tissue culture conditions in DMEM plus 10% FBS. The tissues were then incubated for 3 h with resazurin working solution. Aliquots of medium were placed in microtiter plate wells and read on a microtiter plate spectrophotometer at a wavelength of 590 nm. The data is expressed as the mean ± 1 standard error (SE) relative fluorescent units. Viable cell recovery was determined by trypsin blue exclusion after enzymatic tissue digestion. The cells were also plated and cultured with DMEM plus 10% FBS for 1 week to verify that the chondrocytes were able to adhere and proliferate in vitro.

Biomaterial Testing

Cartilage plugs were also evaluated for permeability by measuring their electrical conductivity to determine if biomaterial changes were occurring during storage. Specimens were prepared by cutting a 5-mm cylindrical plug using a cornal trephine from the stored 8-mm diameter cartilage disc. In the first series of experiments, the superior and inferior surfaces of each cylindrical sample were trimmed using a sledge microtome (Leica SM2400, Nussloch, Germany) after adherence to the microtome stage by freezing to ensure flat, parallel surfaces for contact with the conductivity electrodes. The samples (n = 29) were tested after 0, 1, 7, and 28 days of storage in isotonic phosphate-buffered saline (PBS). In a second series of tests in which samples were tested after 0 and 28 days of storage, the cartilage surfaces were trimmed manually using a sharp blade without freezing. Then conductivity was first tested in isotonic PBS followed by swelling and testing in hypotonic saline (0.2 × PBS). The height of each specimen was measured with an electrical current-sensing micrometer. The method and apparatus for measuring electrical conductivity of tissues was reported previously [21]. Briefly, the conductivity apparatus consists of two stainless steel current electrodes coaxial to two Teflon-coated Ag/AgCl voltage electrodes placed on the top and bottom of a cylindrical nonconductive plexiglass chamber (5 mm diameter). Applying the four-wire method and using a source meter (Model 2400, Keithley Instruments, Inc., Cleveland, OH, USA), the resistance (R) values across the specimens were measured at a low, constant current density of 0.015 mA/cm². The electrical conductivity (χ) values of the specimens were calculated by:

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\chi = \frac{h}{R \times A}
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where h and A are the height and cross-sectional area of the specimen, respectively. All electrical conductivity measurements were performed in PBS at room temperature (22 °C). Electrical conductivity is a material property of biological tissues. Its value is related to the diffusivity of small ions inside the tissue, which depend on tissue composition and structure [22, 23]. Using an electrical conductivity method, the effect of matrix composition on solute permeability has been studied in hydrogels and cartilaginous tissues [24, 25]. In this study, we adopted this method to study the impact of 4 °C storage on cartilage ECM solute permeability. Moreover, the transport of small solutes (e.g. ions, oxygen, and glucose)
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Storage in culture medium provides good cartilage viability and metabolic function for 7 days; however, significant changes were observed in femoral heads as early as 1 week (fig. 1). All mean chondrocyte assessment values were <30% of fresh controls at 28 days. Cartilage plugs tended to perform better after 7 days of storage than the femoral heads and retained significantly higher metabolic activity (mean = 94.5% vs. 70.5%). After 7 days of storage the metabolic assay results were consistently higher than the cell isolation results (fig. 1). After completion of the trypan blue viability counts, the cells were plated and cultured. Representative areas in each culture flask photographed after 1 week in culture revealed similar degrees of confluence (fig. 2).

The prepared disc-shaped specimens employing the microtome (n = 4 for each group) had an average height of 2.49 ± 0.57 mm and a diameter of 5 mm. In the first experiment, fresh cartilage demonstrated a mean conductivity of 12.6 mS/cm, which decreased significantly to 10.7 and 7.8 mS/cm at 7 and 28 days of storage [27], respectively. However, further experiments did not demonstrate significant decreases during storage (not shown), and there were no significant differences when data from these experiments were combined (n = 29, fig. 3). Therefore, the cartilage plugs failed to demonstrate consistent significant changes in electrical conductivity as a measure of permeability over 28 days of storage.

We were however concerned that the method employed to mount the plugs, i.e. freezing to a chuck to trim the tissue, may have obscured differences between the experimental and control groups, particularly because we had seen differences in the first of the four experiments above. Samples from four individual porcine donors were then prepared without freezing, and the superficial layer and deep layer was trimmed from each end of the plug manually. The average height after trimming was 1.55 ± 0.35 mm (n = 20). Significant differences were observed for plugs tested in isotonic saline (p = 0.007) and hypotonic saline (p < 0.0001) groups using a paired t-test.
We found, in agreement with the literature, that storage in DMEM culture medium with 10% FBS provides good cartilage viability for 7 days. The metabolic assay used to determine the viability of the chondrocytes within intact cartilage provided higher cell viability values at 7 days than the cell isolation studies with trypan blue. In contrast by 28 days the results using the two assays were similar with less than 30% cell viability (fig. 1). Our interpretation of these results is that many of the chondrocytes may have been damaged at 7 days, resulting in loss during the cell isolation procedures employing collagenase required prior to the determination of cell viability using trypan blue. This may also explain concerns previously expressed regarding the use of fluorescent assays for cartilage storage studies [19]. It is possible that the metabolic assay at this time point is an accurate measure of potential viability if the tissues were implanted rather than subjected to collagenase digestion. The trypan blue-excluding cells from fresh and both 7- and 28-day-stored tissues were able to proliferate and resulted in qualitatively similar cultures after 1 week in vitro (fig. 2).

Bisected femoral heads and much smaller cartilage plugs were compared to evaluate the impact if any of cartilage volume on cell survival. There was a marked tendency for cartilage plugs to demonstrate higher viability values after 7 days of storage than the femoral heads, but it was only statistically significant for metabolic activity (fig. 1). The results after 28 days were similar. These results confirm the use of cartilage plugs for further studies to compare the impact of different storage solutions and additives on chondrocyte survival. This will include comparison of hypothermic storage solutions, such as the University of Wisconsin solution (UW), employed for organ and tissue storage and more complex culture media. UW is an example of ‘intracellular-type’ preservation solutions which are typically hypertonic and formulated to restrict the passive exchange of water and ions during hypothermia-induced inhibition of cell membrane pumps [reviewed in 35]. An intracellular-type solution usually includes a non-permeating, impermeant anion such as lactobionate or gluconate to partially replace chloride ions in the extracellular space. This provides osmotic support to balance the intracellular oncotic pressure generated by cytosolic macromolecules and their associated counter-ions locked inside the cell. In contrast, saline and culture media are extracellular-type solutions. They are isotonic with a plasma-like complement of ions that mimics the normal extracellular environment of cells. Tissue culture media contain a more complete complement of ions, amino acids, and other metabolites that mimic the extracellular composition of plasma. Most published studies on cartilage hypothermic storage have employed extracellular-type solutions. Onuma et al. [12] compared DMEM, saline, EuroCollins solution, and UW solution to determine which provided the best hypothermic preservation of rat osteochondral tissues. They concluded on the basis

![Fig. 3. Impact of hypothermic storage of cartilage on electrical conductivity in isotonic saline. In these experiments the cartilage plug was prepared using freezing stage. There were no statistically significant changes in permeability, p = 0.139, the data is expressed as the mean ± 1 SD, n = 29.](image)

![Fig. 4. Comparison of fresh control and 28-day-stored cartilage plug electrical conductivity under isotonic (open bars) and hypotonic (patterned bars) conditions. In these follow-up experiments the cartilage plugs were trimmed without freezing stage and conductivity was assessed first in isotonic saline (1 × PBS) and then in hypotonic saline (0.2 × PBS). The data is expressed as the mean ± 1 SD and * indicates significant differences at p < 0.05, n = 20.](image)
of two assays and histology that UW solution was the most suitable. Teng et al. [18] clearly demonstrated the positive impact of more complex culture media formulation upon chondrocyte survival. There are other indications in the literature that media supplementation or modification may promote chondrocyte survival. Removal of FBS from storage solutions is also an issue for tissue bank products due to FBS batch variation and the associated health risks [18, 36]. Extensive research is needed to determine whether intracellular- or extracellular-type solutions are best for chondrocyte preservation in cartilage and to define the optimal solution composition.

Cartilage tissue is comprised of a solid and fluid phase, and it may be treated as a biphasic material. Cartilage executes its role in the body to absorb and distribute joint stresses based on this mechanism. Therefore, the permeability characteristic is an important material property of cartilage that is usually overlooked in contrast to more commonly studied tensile or compressive parameters. Studies of such biomechanics parameters in the literature have consistently failed to demonstrate changes in cold stored cartilage. In our first series of experiments employing isotonic saline during evaluation, we did not observe consistent changes in permeability. However, we saw changes in the first experiment [27], contributing to the negative data in figure 3, and we previously observed extracellular matrix damage in frozen articular cartilage [37]. Therefore, in order to rule out the possibility that ice formation during trimming of the cartilage plugs had changed sample permeability, we performed another series of experiments comparing 0 and 28 days of storage (n = 20). First the samples were tested in isotonic saline (1 × PBS) and then retested in hypotonic (0.2 × PBS) saline. Hypotonic saline was used because we hypothesized that it might render the assay system more sensitive to hypothetical ECM changes. The results (fig. 4) demonstrated statistically significant changes during testing in both isotonic and hypotonic saline, and the differences were greater in hypotonic saline than in isotonic saline. Hypotonic solution made the tests more sensitive due to increased tissue swelling which magnifies changes in the highly charged ECM [21]. Further experiments to determine the time course of these permeability changes are needed. In addition, laser scanning microscopy [37] or RAMAN spectroscopy may detect changes in collagen structure.

In conclusion, 28-day storage of cartilage results in loss of chondrocyte viability and ECM permeability. Storage methods for clinical cartilage utilization should be carefully evaluated because the tissue may be experiencing clinically significant deterioration over time. We plan further investigation of cartilage cell and biomaterial properties during storage combined with storage solution formulation optimization. Improved storage solutions could result in increased utilization of banked allogeneic cartilage for reconstruction of articular cartilage defects and possibly storage and distribution of tissue-engineered cartilage.

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Disclosure Statement

Dr. Brockbank is an owner and employee of Cell and Tissue Systems. Mrs. Chen is also an employee of the company.

References


