Enhancing Osteochondral Allograft Viability

Effects of Storage Media Composition

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Abstract Osteochondral allograft transplantation is a well-accepted treatment for articular cartilage damage. However, chondrocyte viability declines during graft storage, which may compromise graft performance. We first tested the hypothesis that the composition of commonly used storage media affects the viability of articular chondrocytes over time; we then tested the hypothesis that the addition of insulin growth factor-1 or the apoptosis inhibitor ZVAD-fmk could enhance the storage properties of serum-free media. Bovine osteochondral grafts were stored at 4°C in lactated Ringer's, Dulbecco's modified eagle's media (DMEM), DMEM supplemented with either insulin growth factor-1 or ZVAD-fmk, and a commercial storage media. Chondrocyte viability in lactated Ringer's declined rapidly to 20.4% at 2 weeks. Viability in DMEM declined more slowly to 54.8% at 2 weeks and 31.2% at 3 weeks. Viability in commercial storage media was 83.6% at 3 weeks and 44.8% at 4 weeks. Viability was increased in DMEM + insulin growth factor-1 (56.4%) and DMEM + ZVAD (52.4%) at 3 weeks compared with DMEM alone. These results confirm the hypotheses that media composition greatly influences chondrocyte viability

during cold storage and that insulin growth factor-1 and ZVAD improve the storage properties of DMEM.

Introduction

The treatment of articular cartilage injury remains one of the most difficult challenges facing orthopaedic surgeons. One treatment option that is particularly well suited for larger injuries is osteochondral allograft transplantation. The use of fresh or cold-stored osteochondral allografts has the theoretical advantage of transplanting viable chondrocytes that can maintain the cartilage tissue. However, a number of studies document cold storage reduces the viability of chondrocytes in human [2, 4, 10, 12, 13, 19] and animal [11, 15, 17, 18] osteochondral tissues. Although results vary substantially among these studies, there is a general consensus that chondrocyte viability declines with time in storage and that the rate of decline is influenced by the composition of the storage media.

The precise mechanisms contributing to decreased viability are not well understood. Recent data suggest some chondrocyte death may be the result of apoptosis or programmed cell death [14]. At present, osteochondral allografts are most commonly stored in lactated Ringer's (LR) or "minimal" cell culture media such as Dulbecco's modified eagle's medium (DMEM). Other agents included in some commercial storage media include fetal bovine serum (FBS), buffers, sugars, additional salts, and amino acids. It is generally believed that chondrocyte viability is improved in media with greater nutritional content; however, research that directly compares chondrocyte viability in these different media is limited.

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We therefore tested the hypothesis that the composition of the media used during cold storage of osteochondral grafts would alter the viability of articular chondrocytes over time. Second, we tested the hypothesis that the addition of recombinant insulin growth factor-1 (IGF-1) and/or the apoptosis inhibitor ZVAD-fmk to cell culture media would increase the survival of chondrocytes during cold storage of osteochondral grafts.

Materials and Methods

We harvested the distal femurs of young adult cows, screened them for macroscopic evidence of damage, and then split each into medial and lateral condyle grafts. Grafts were submerged in one of five different storage media: (1) LR (Baxter, Deerfield, IL); (2) DMEM (UCSF Cell Culture Facility); (3) DMEM supplemented with recombinant IGF-1 (100 ng/mL) (Leinco Technologies Inc, Ballwin, MO); (4) DMEM supplemented with a potent inhibitor of caspase-dependent apoptosis, ZVAD-fmk (50 μ M) (Calbiochem, San Diego, CA); and (5) a proprietary commercial storage media composed of DMEM/F12 media supplemented with FBS, HEPES buffer, amino acids, vitamins, sugars, and salts (Jim Shock, Musculoskeletal Transplant Foundation (MTF), NJ, personal communication). These are in approximate order of nutritional completeness. All media contained penicillin (100 U/mL), streptomycin (100 U/mL), and Fungizone (1.5 μ g/mL) (UCSF Cell Culture Facility). Grafts were maintained at 4°C in sealed containers equilibrated with room air without changing media. On the day of harvest and at weekly intervals thereafter, chondrocyte viability (dependent variable) was measured as described subsequently. Data were collected from two independent experiments for MTF media, and from a minimum of three independent experiments for all other media. Each experiment was performed using grafts from different animals. For each graft, four to eight cartilage samples per time point per condition were harvested. Power analysis computed a minimum sample size of nine for a two-tailed t-test with an effect size of 0.75, alpha of 0.05, and power of 0.8.

At 0, 1, 2, 3, and 4 weeks, full-thickness articular cartilage samples were taken using 4-mm dermal biopsy punches. A minimum of 8 mm was maintained from areas sampled at previous time points to avoid confounding effects on cell viability. The cylindrical samples were halved and mounted on plastic blocks. We submerged samples in a bath of LR and sectioned them into 100- μ m slices using a vibratory microtome (Vibratome, St Louis, MO). Sections were taken near the center of the halved plug to minimize artifact from sample manipulation.

Cell viability was assayed using the Live/Dead[®] Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR), which assays cell viability effectively in dense human connective tissue, including cartilage [6]. Live cells were stained with calcein-AM and dead cells with EthD-1. Samples were incubated for 30 minutes at room temperature with a solution of 1 μ mol EthD-1 and 1 μ mol calcein-AM. These concentrations were modified slightly from the manufacturer's protocol to optimize the sensitivity and specificity of the assay. This technique provides consistent and reproducible values for chondrocyte viability and was not subject to the types of cell loss artifacts reported previously when Live/Dead staining is used for analysis of frozen sections [7].

We captured fluorescence microscopic images with an Axiocam digital camera (Carl Zeiss, Thornwood, NY) at a resolution of 1 megapixel. For each sample, we screened Vibratome sections for processing artifacts and a representative image was selected for analysis. Cells were counted from a 1250- μ m wide by 1000- μ m deep area near the center of each slice. Semiautomated data collection and analysis were carried out using Adobe Photoshop (Adobe Systems Inc, San Jose, CA) and a public domain Java image processing program (ImageJ; National Institutes of Health, Bethesda, MD).

We determined mean percent live cells $\pm$ standard error of the mean. We compared viability between the five treatment groups using a paired Student's t test.

Results

We observed differences in viability at all time points with greater viability in more nutritionally complete media (Table 1). In osteochondral grafts stored in LR, chondrocyte death occurred throughout all zones of the articular cartilage (Fig. 1). Chondrocyte viability decreased rapidly to $58.0\% \pm 8.3\%$ at week 1 and $20.4\% \pm 3.6\%$ at week 2 (Fig. 2). When grafts were stored in DMEM, chondrocyte viability decreased more slowly to $67.3\% \pm 5.1\%$ at week 1 and $54.8\% \pm 4.7\%$ at week 2 (Fig. 2). Analysis of later time points demonstrated $31.2\% \pm 3.5\%$ viability at week 3 and $14.6\% \pm 4.1\%$ at week 4. Osteochondral grafts stored in proprietary commercial (MTF) media supplemented with FBS, amino acids, vitamins, sugars, and salts had higher chondrocyte viability at all time points compared with DMEM: $93.8\% \pm 0.7\%$ ($p = 0.07$), $86.3\% \pm 1.5\%$ ($p = 0.0017$), $83.6\% \pm 1.2\%$ ($p < 0.0001$), and $44.8\% \pm 4.3\%$ ($p = 0.0004$) at weeks 1 through 4, respectively (Fig. 2).

Chondrocyte viability in grafts stored in DMEM + IGF-1 was $69.8\% \pm 3.7\%$ at week 1, $58.6\% \pm 4.9\%$ at week 2, $56.4\% \pm 4.3\%$ at week 3, and $5.9\% \pm 0.6\%$ at week 4

Table 1. Mean cell viability at each time point

Storage medium	Week 0	Week 1	Week 2	Week 3	Week 4
Lactated Ringer's	92.1% $\pm$ 1.5%	58.0% $\pm$ 8.3%	20.4% $\pm$ 3.6%		
DMEM	92.7% $\pm$ 0.6%	67.3% $\pm$ 5.1%	54.8% $\pm$ 4.7%*	31.2% $\pm$ 3.5%	14.6% $\pm$ 4.1%
MTF media	90.7% $\pm$ 1.8%	93.8% $\pm$ 0.7%	86.3% $\pm$ 1.5% [‡]	83.6% $\pm$ 1.2%	44.8% $\pm$ 4.3% [§]
DMEM plus IGF-1 (100 ng/mL)	92.1% $\pm$ 0.8%	69.8% $\pm$ 3.7%	58.6% $\pm$ 4.9%	56.4% $\pm$ 4.3%	5.9% $\pm$ 0.6%
DMEM plus ZVAD-fmk (50 μ M)	93.2% $\pm$ 1.0%	72.6% $\pm$ 7.3%	62.5% $\pm$ 6.5%	52.4% $\pm$ 6.0% [†]	16.1% $\pm$ 4.1%

Values are mean $\pm$ standard deviation; * p < 0.0001 compared with lactated Ringer's; [†] p = 0.002 compared with DMEM; [‡] p = 0.0017 compared with DMEM; [§] p = 0.0004 compared with DMEM; ^{||} p < 0.0001 compared with DMEM; DMEM = Dulbecco's modified eagle's medium; MTF = Musculoskeletal Transplant Foundation; IGF-1 = insulin growth factor-1.

Fig. 1 Representative photomicrographs of cartilage biopsy specimens after Live/Dead staining demonstrate declining viability over 2 weeks of cold storage in lactated Ringer's. Numbers indicate mean

percent viability at each time point. Live cells are stained green and dead cells are stained red. Scale bar = 250 μ m. The superficial layer is oriented toward the top of the page.

Fig. 2 Storage media markedly influences articular chondrocyte viability of osteochondral allografts during cold storage. Bovine osteochondral grafts were stored in lactated Ringer's (LR), Dulbecco's modified eagle's medium (DMEM), and DMEM/F12 supplemented with fetal bovine serum, HEPES buffer, amino acids, vitamins, sugars, and salts. Data are presented as mean percent live $\pm$ standard error of mean. MTF = Musculoskeletal Transplant Foundation.

(Figs. 3, 4). Viability at week 3 was higher (p < 0.0001) in DMEM + IGF-1 compared with DMEM alone. Chondrocyte viability in grafts stored in DMEM + ZVAD-fmk was 72.6% $\pm$ 7.3% at week 1, 62.5% $\pm$ 6.5% at week 2,

52.4% $\pm$ 6.0% at week 3, and 16.1% $\pm$ 4.1% at week 4 (Figs. 3, 4). Viability at week 3 was higher (p = 0.002) in DMEM + ZVAD-fmk compared with DMEM alone.

Discussion

Survival of chondrocytes in cold-stored osteochondral grafts is believed to enhance the long-term performance of these tissues in reconstructive procedures. However, declining chondrocyte viability during the storage process compromises the potential advantage of transferring viable cells during the transplantation procedure. We therefore tested two hypotheses: (1) the composition of the media used during cold storage of osteochondral grafts would alter the viability of articular chondrocytes over time; and (2) the addition of recombinant insulin growth factor-1 (IGF-1) and/or the apoptosis inhibitor ZVAD-fmk to cell culture media would increase the survival of chondrocytes during cold storage of osteochondral grafts.

Several limitations of this study should be considered in interpreting our results. First, we used bovine osteochondral grafts rather than human grafts. Second, our data were limited to chondrocyte viability without any measures of metabolic function of those viable cells. Third, the exact composition of the commercial storage media provided by the Musculoskeletal Transplant Foundation is proprietary; therefore, the contribution of each component is difficult to

Fig. 3 Representative photomicrographs of cartilage biopsy specimens after Live/Dead staining are shown. Specimens were taken from grafts stored in Dulbecco's modified eagle's medium (DMEM), DMEM + insulin growth factor-1 (IGF-1; 100 ng/mL), or DMEM + ZVAD-fmk (50 μ M). Numbers indicate mean percent viability at each time point. Live cells are stained green and dead cells are stained red. Scale bar = 250 μ M. The superficial layer is oriented toward the top of the page.

Fig. 4 Articular chondrocyte viability of osteochondral allografts stored in Dulbecco's modified eagle's medium (DMEM) with and without insulin growth factor-1 (IGF-1; 100 ng/mL) or the apoptosis inhibitor ZVAD-fmk (50 μ M) is shown graphically. Data are presented as mean percent live $\pm$ standard error of mean. The addition of either IGF-1 or ZVAD-fmk increased ($p < 0.001$, denoted with asterisks) viability after 3 weeks of cold storage.

assess. Nevertheless, our experimental data yielded a substantial number of important findings.

Lactated Ringer's is an isotonic solution consisting of electrolytes and lactate but lacking any nutrients to sustain cellular growth. Historically, LR has been used for short-term graft storage, but its use in cartilage allograft banking has been largely discontinued in favor of other forms of nutrient-containing storage media. Our data confirm that the rapid decline in viability precludes the use of LR for longer-term storage.

One previous study of human osteochondral grafts demonstrated grafts stored in LR have a lower percent of viable chondrocytes in comparison to grafts stored in

standard cell culture media [2]. DMEM is a commonly used "minimal" cell culture media that contains basic nutrients capable of supporting cellular viability. Typical "minimal" cell culture media contain amino acids, salts, and glucose [19]. Our data demonstrate the decline in chondrocyte viability of bovine osteochondral grafts stored in DMEM occurred at a more gradual rate in comparison to grafts stored in LR. However, mean viability still declined by approximately half after 2 weeks in cold storage and by greater than 85% after 4 weeks. In the United States, 2 weeks is typically the minimum amount of time required to complete testing of osteochondral grafts for microbial contamination. Therefore, these findings raise the question of whether "minimal" cell culture media such as DMEM is optimal for prolonged cold storage of osteochondral grafts. The extremely low chondrocyte viability observed in our study at later time points contrasts somewhat with data from similar studies using human osteochondral tissue that reported a chondrocyte viability from 27% to 83% after 28 days of cold storage [2, 13, 19]. Bovine articular cartilage differs from human articular cartilage in terms of mechanical properties, cartilage thickness, and cell density [1, 16]. Therefore, some differences in its response to cold storage are not unexpected. Another contributing factor could be the studies with human specimens included storage media changes every 2 to 7 days, whereas no media changes were made in the present study in accordance with typical commercial storage protocols.

The storage media provided by a commercial tissue bank (Musculoskeletal Transplant Foundation) is based on DMEM/F-12 and contains FBS, HEPES buffer, antibiotics, and supplemental amino acids, vitamins, sugars, and salts. As expected, chondrocyte survival during cold storage in this media was higher than in either LR or DMEM. However, because of the complexity of the media and the unavailability of detailed information regarding the specific components, it is unclear which components are most

responsible for the observed increase in chondrocyte survival. One component that likely contributed to improved chondrocyte viability was FBS. In a recent study on human osteochondral grafts, the addition of FBS augmented cell viability [13]. However, the inclusion of FBS is not without disadvantages that include lack of consistency among different lots of serum, potential immunologic reactions, and potentially serious or even lethal infectious disease transmission [13].

Theoretically, single well-characterized drugs such as recombinant growth factors could substitute for FBS. IGF-1 is an attractive candidate based on its ability to decrease chondrocyte cell death in other models of cartilage viability. IGF-1 is a key anabolic growth factor in normal cartilage tissue [9]. We found chondrocyte viability in bovine osteochondral grafts declined at a slower rate in storage media supplemented with IGF-1 over the first 3 weeks of cold storage. However, no difference was observed after 4 weeks of storage. Because the media was not changed in these experiments, one possible explanation is the IGF-1 was degraded or otherwise inactivated during the storage period. This problem potentially could be overcome by repeated addition of IGF-1 throughout the storage period. However, any benefits of IGF-1 must be weighed against its primary disadvantage, cost. It is also likely additional, and potentially costly, agents would be necessary to fully substitute for FBS; therefore, the economic feasibility of developing a “defined” storage media that is equivalent or superior to media used commercially remains an unanswered question.

A recent study reported multiple key mediators of apoptosis are upregulated during cold storage of human osteochondral tissues [14]. Theoretically, blockade of these mediators of apoptosis could decrease cell loss during cold storage. IGF-1 inhibits chondrocyte programmed cell death, which may contribute to its beneficial effects noted in our experiments [5, 8]. The prototypical apoptosis inhibitor ZVAD-fmk is a potent irreversible inhibitor of multiple caspases and has been used in previous studies of apoptosis inhibition in cartilage [3, 5]. The addition of ZVAD-fmk to DMEM increased chondrocyte viability compared with DMEM alone; however, we did not formally demonstrate the mechanism was prevention of apoptosis.

Our experiments using bovine osteochondral tissue provided data comparable to previous studies using human tissues; therefore, we believe bovine osteochondral grafts are a reasonable substitute for valuable human grafts in experiments testing parameters of graft storage. This study supports others that generally show an association between increased nutritional content of storage media with enhanced chondrocyte survival. The addition of recombinant IGF-1 and ZVAD-fmk to DMEM improved chondrocyte survival providing proof of concept for the

development of a “defined” storage media that could exclude FBS with its associated problems. Extensive research will be needed to define the optimal components of a “defined” storage media because the combinatorial possibilities are essentially limitless. Nevertheless, we believe the end objective appears attainable.

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