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The effect of chondrocyte cryopreservation on cartilage engineering

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Abstract

Chondrocytes were collected from the stifle joints of four pigs to study the effect of cryopreservation on the chondrogenic potential of chondrocytes. Half of the cells were cryopreserved for 3 months. Polyglycolic acid scaffolds were cultured with fresh or cryopreserved chondrocytes for 4 weeks. Cell morphology and the quality of engineered tissue were evaluated by scanning electron microscopy, histopathology and biochemical methods. More cells attached to scaffolds at 48 h when fresh chondrocytes were seeded. At 4 weeks, the numbers of cells, DNA and collagen II were greater in constructs engineered by fresh cells. However, the collagen II/DNA ratio did not differ between the two groups. More matrix was identified on a scanning electron microscope and by histopathology in the fresh group. Cartilage engineered with cryopreserved chondrocytes may contain less matrix and fewer cells. These findings most likely resulted from a lack of cell attachment on the matrix secondary to cryopreservation. Future studies are needed to further evaluate the mechanism by which cryopreservation may affect chondrocyte attachment.
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Keywords: Chondrocyte; Cryopreservation; Cartilage; Tissue engineering; Bioreactor

Introduction

The limited potential of chondrocytes for proliferation and the avascular nature of cartilage contribute to poor healing in adult cartilage, eventually leading to osteoarthritis (Vunjak-Novakovic et al., 1999; Glowacki, 2000; Lu et al., 2001; Tew et al., 2001). Autologous chondrocyte transplantation/implantation (ACT) is one of the latest methods for cartilage resurfacing in humans and horses (Hendrickson et al., 1994). In this technique, autologous chondrocytes are harvested from the patient, expanded, then grafted into the defect during a second surgery (Brittberg et al., 2001; Minas, 2001; Marlovits et al., 2005). However, transplantation of freshly expanded cells currently requires a high degree of flexibility regarding the timing of re-implantation. Therefore, cryopreservation of autologous chondrocytes has been used occasionally to facilitate the scheduling of the second surgery (Hendrickson et al., 1994; Minas, 2001; Velikonja et al., 2001; Drobnic et al., 2002).

In an attempt to develop reliable methods to produce cartilaginous tissue in vitro, articular chondrocytes have been cultured on three-dimensional (3D) biodegradable scaffolds in tissue culture bioreactors. Three-dimensional matrices promote the accumulation of synthesized matrix molecules and prevent the loss of cell phenotype compared to monolayer culture systems. Although the ideal scaffold for development of ex vivo cartilage remains undetermined, there is evidence to suggest that chondrocytes may attach preferentially to mesh-based scaffolds (Pei et al., 2002). Among those, a polyglycolic acid (PGA) mesh has been used extensively as a resorbable substrate on which chondrocytes produce implant-free cartilage (Vunjak-Novakovic et al., 1998, 1999; Coutts et al., 2001; Lu et al., 2001; Blunk et al., 2002; Griffon et al., 2005). The long-term goal

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of studies like these is to optimize cartilage engineering for clinical applications (Freed et al., 1993a,b; Hunziker and Kapfinger, 1998; Mainil-Varlet et al., 2001; Schaefer et al., 2002). However, studies in cartilage engineering typically involve fresh chondrocytes harvested immediately after euthanasia of very young animals (Freed et al., 1997, 1998; Vunjak-Novakovic et al., 1998), a procedure not readily applicable to the clinical situation. However, the harvesting of cartilage from clinical patients is limited by availability and lack of predictability. Cryopreservation of chondrocytes would facilitate the scheduling of experiments on chondrocytes harvested from clinical patients.

Although cryopreservation would be advantageous for clinical and experimental purposes, preserving viability and maintaining functional integrity of chondrocytes is crucial in order to optimize use of donor cells and restoration of cartilage (Perka et al., 2000). Studies evaluating the influence of cryopreservation on chondrogenic potential of chondrocytes are, however, limited and lack objective characterization of the matrix constituents (Schachar et al., 1989; Bujia et al., 1992; Falsafi and Koch, 2000; Perka et al., 2000; Gorti et al., 2003). In addition, chondrocytes in these studies were generally cultured in monolayer rather than 3D systems, leading to cellular dedifferentiation (Schachar et al., 1989; Bujia et al., 1992). Another limitation of these studies arises from their short cryopreservation periods (1–7 days) (Schachar et al., 1989; Bujia et al., 1992), which has limited use for cell banking purposes or clinical applications of chondrocyte preservation. For instance, cells harvested for clinical application of the ACT technique occasionally have been preserved anywhere from 6 weeks to 2 years before scheduling of elective surgery (Brittberg et al., 1994).

In the current study, we investigated the effect of cryopreservation for up to 3 months on the viability, proliferation and collagen II production of chondrocytes cultured in a 3D environment under dynamic conditions. The 3 months of cryopreservation in this study may better simulate the clinical condition of ACT and chondrocyte banking than the shorter periods used in previously reported studies.

Materials and methods

Scaffolds

All scaffolds consisted of a non-woven mesh made of 13-μm diameter PGA fibers (Synthecon). A total of 44 scaffolds, divided into two equal groups, were used in two phases of the study.

Cell isolation

The project was approved by the University of Illinois Institutional Animal Care and Use Committee.

Cartilage was aseptically collected from the stifle joints of four 3–4-week-old pigs as previously described (Griffon et al., 2005). The cartilage was minced and digested according to standard methods (Freed et al., 1993a,b). The culture medium used in the study was Dulbecco's modified Eagle's medium (DMEM; Freed et al., 1993a,b). Cell number and viability were evaluated via trypan blue exclusion. Chondrocytes were suspended in culture medium to obtain a cell suspension containing 20×10^6 viable cells/mL. The suspension was divided into two groups: Half the cells were immediately cultured in the first phase of the study, while the other half were cryopreserved for 3 months prior to inclusion in the second phase of the study.

Cryopreservation and expansion

Half of the harvested cells were preserved in a medium composed of 10% dimethylsulfoxide (DMSO) (Sigma–Aldrich Inc.) in 40% fetal bovine serum and 50% DMEM. Eight million viable cells were suspended in 1 mL of medium prior to transfer into each cryogenic vial (Corning), followed by a two-staged cooling preservation (Mazur et al., 1972; Tomford et al., 1984; Freshney, 2000; Hay et al., 2000). Cryovials were placed in a cryofreezing container (Nalgene, RPI Corp) in a −80 °C freezer to decrease the temperature at a rate of 1 °C/min for the first 12 h. Cryovials were then transferred into liquid nitrogen (−196 °C) and preserved for 3 months.

Three months after preservation, frozen cell suspensions were thawed, diluted in culture medium and centrifuged three times (Gorti et al., 2003). After determining the rate of cell recovery, each cell suspension containing 1×10^6 viable cells was transferred to a 75 cm^2 culture flask (BD Falcon Biosciences), expanded and passaged three times to obtain a minimum of 120 million viable cells.

Cell seeding and culture

Twenty-two scaffolds were loaded with chondrocytes isolated from each pair of pigs. Half ($n = 11$) of the scaffolds were seeded with fresh cells, while the other half received cryopreserved cells. The experiment was repeated with cells pooled from two other pigs. Scaffolds were seeded with a density of 5×10^6 live cells/scaffold using a spinner flask technique (Vunjak-Novakovic et al., 1999; Griffon et al., 2005).

Each spinner flask was filled with 120 mL of a cell suspension containing 55×10^6 viable cells (Schaefer et al., 2002), supplemented with 5% CO_2 and incubated at 37 °C. One scaffold from each flask ($n = 4$) was fixed for scanning electron microscopic (SEM) evaluation 48 h after seeding. The 10 scaffolds remaining in each flask were transferred into a rotating bioreactor (Synthecon Inc.) filled with the same culture medium and cultured at 37 °C with 5% CO_2 for 4 weeks.

Evaluation of the constructs

Seeding phase

Forty-eight hours after seeding, one scaffold from each spinner flask was fixed prior to SEM evaluation, using a previously described technique (Griffon et al., 2005). Cells were counted in 10 fields of 115 × 80 μm (at 800 times magnification).

Evaluation at 4 weeks

After 4 weeks of culture, samples randomly selected for biochemical analysis were weighed before and after lyophilization to determine their water content. Constructs in each phase of the study were divided for DNA quantification using Hoechst 33258 (Kim et al., 1988). Collagen II content was determined by enzyme linked immunosorbent assay (Native Type II Collagen Detection Kit, Arthrogen-CIA). For histopathological examination, samples were stained with hematoxylin and eosin (HE). Examination of the constructs via SEM was also performed to subjectively evaluate the quality of the extracellular matrix.

Data analysis

The number of cells counted in each scaffold via SEM examination and biochemical parameters measured after 4 weeks of culture were compared between constructs engineered by fresh and cryopreserved chondrocytes using the Student's t test ($P \leqslant 0.05$).