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The viability and proliferation of human chondrocytes following cryopreservation

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Abstract

Human articular cartilage samples were retrieved from the resected material of patients undergoing total knee replacement. Samples underwent automated controlled freezing at various stages of preparation: as intact articular cartilage discs, as minced articular cartilage, and as chondrocytes immediately after enzymatic isolation from fresh articular cartilage. Cell viability was examined using a LIVE/DEAD assay which provided fluorescent staining. Isolated chondrocytes were then cultured and Alamar blue assay was used for estimation of cell proliferation at days zero, four, seven, 14, 21 and 28 after seeding. The mean percentage viabilities of chondrocytes isolated from group A (fresh, intact articular cartilage disc samples), group B (following cryopreservation and then thawing, after initial isolation from articular cartilage), group C (from minced cryopreserved articular cartilage samples), and group D (from cryopreserved intact articular cartilage disc samples) were 74.7% (95% confidence interval (CI) 73.1 to 76.3), 47.0% (95% CI 43 to 51), 32.0% (95% CI 30.3 to 33.7) and 23.3% (95% CI 22.1 to 24.5), respectively. Isolated chondrocytes from all groups were expanded by the following mean proportions after 28 days of culturing: group A ten times, group B 18 times, group C 106 times, and group D 154 times.

This experiment demonstrated that it is possible to isolate viable chondrocytes from cryopreserved intact human articular cartilage which can then be successfully cultured.

Over the last ten years there has been significant interest in the application of autologous chondrocyte implantation for treating defects of articular cartilage in joints.^{1,2} The United Kingdom National Institute for Clinical Excellence (NICE) highlighted the application of cryopreservation as a potentially useful method for allowing tissue to be stored until

required for culture or implantation.³ Cryopreservation of isolated chondrocytes has been previously studied in human and animal specimens, and there is one successful report of its use in autologous chondrocyte implantation.^{2,4-7} However, there is continued debate about whether these methods can be used successfully with whole pieces of human cartilage tissue.^{6,7} There has been little investigation into the effect of cryopreservation on the process of chondrocyte culture and its implications in treatment using autologous chondrocyte implantation.^{2,5}

The aim of this study was to establish whether viable human chondrocytes can be isolated from cryopreserved cartilage, and whether the isolated chondrocytes can subsequently be cultured to expand their numbers.

Materials and Methods

Following ethical approval, specimens of resected joint surface were collected from 15 patients undergoing total knee replacement. There were ten women and five men with a mean age of 73.3 years (52 to 91). The specimens were immersed in serum-free Dulbecco's minimal essential medium (DMEM) and initially maintained at 4°C. Using a custom-made punch, 12 cores from each patient, each 5 mm in diameter, were harvested from regions of articular cartilage which appeared macroscopically normal in each specimen. The subchondral bone was removed from each sample to leave a disc of cartilage approximately 5 mm × 2 mm. From each specimen, three discs were assigned to each of four sample groups: A (control), chondrocytes were isolated from the cartilage disc and cell viability was assessed; B, isolated chondrocytes from the cartilage disc were cryopreserved and stored in liquid nitrogen at -196°C for 24 hours before being thawed and assessed for viability; C, the disc was minced with a scalpel to yield pieces of approximately 1 mm³ which were also cryopreserved and stored in liquid nitrogen, at -96°C for 24 hours before being thawed. Chondrocytes were then isolated and assessed for viability; D, the whole disc was cryopreserved and stored in liquid nitrogen at -196°C for 24 hours before being thawed. Chondrocytes were then isolated and assessed for viability.

Cryopreservation protocol

A Planer Biomed KRYO 10 series (Planer plc, Sunbury, United Kingdom) was used for programmed step-cooling of all test samples. The programme used for the studies was one that proved effective for the retention of viability of the cartilage cells in pilot studies. This procedure involved (1): cooling from room temperature to 4°C, at a rate of -5°C/min, (2): cooling from 4°C to -30°C, at a rate of 1°C/min, (3): cooling from -30°C to -80°C, at a rate of -2°C/min, (4) immediate transference of the samples to a liquid nitrogen storage tank. The cryopreservative medium used was 10% dimethyl sulfoxide (DMSO) in 90% fetal calf serum (FCS). For cryopreservation, samples from both the diced and the intact cored disc groups were placed into cryogenic vials (Nalgene, Nalge Company, New York, New York) containing 1 ml cryogenic medium and incubated for 30 minutes at room temperature to allow penetration of a cryoprotective agent before being subjected to the cryopreservation programme.

Isolation of chondrocytes

After optimisation of the isolation technique, collagenase type II (Gibco BRL, Invitrogen Ltd, Paisley, United Kingdom) at a concentration of 400 units/ml was used to isolate chondrocytes from diced articular cartilage, with incubation at 37°C for 16 hours. This method was used for samples regardless of whether the cartilage tissue had been cryopreserved or not.

Viability assay

The cells were washed twice in phosphate-buffered saline (PBS) to remove any serum. Samples were then stained using a LIVE/DEAD assay (Molecular Probes, Leiden, The Netherlands). This kit contains two fluorescent dyes, calcein AM to stain living cells green, and ethidium homodimer-1 (Ethd-1) to stain damaged or dead cells red. The samples were stained using 4 μ M calcein and 2 μ M Ethd-1 (final concentration) in PBS for 30 minutes at 37°C in an incubator. After staining, samples were rinsed twice in PBS to remove excess dye, observed by an epifluorescent microscope (Zeiss Axioplan, Carl Zeiss Ltd, Oberkochen, Germany). Cell viability was measured by assessing the proportion of living cells isolated using disposable cell counting chambers (Fast Read 102, ISL, Paignton, United Kingdom).

Culturing cryopreserved cells and measuring proliferation

Chondrocytes isolated from each sample group were seeded into 24-well plates (5×10^4 cells per well) with 20% FCS DMEM and incubated at 37°C. The medium was changed every three days. Cell proliferation was measured at zero, four, seven, 14, 21 and 28 days using an Alamar blue assay for cell numbers. The culture medium was removed and fresh serum-free DMEM containing 5% Alamar blue was added before culturing at 37°C in an incubator for two hours. At that point, the relative fluorescence units of the Alamar blue-containing medium were measured using a microplate reader (SPECTRAMax GEMINI Molecular Devices, Sunnyvale, California) and analysis software SoftMAXPRO (Molecular Devices). Standard numbers of viable chondrocytes were used to prepare a standard curve for derivation of the relationship of the relative fluorescence units to the cell number.

Statistical analysis

Data are presented as means with 95% confidence intervals (CI). GraphPad InStat statistical software (GraphPad Software, LaJolla, California) was used to perform analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons tests, with $p < 0.05$ being regarded as significant.

Results

The mean percentage viability of isolated chondrocytes from groups A, B, C and D were 74.7% (95% CI 73.1 to 76.3), 47.0% (95% CI 43 to 51), 32.0% (95% CI 30.3 to 33.7) and 23.3% (95% CI 22.1 to 24.5), respectively (Fig. 1). The results for all cryopreserved specimens were significantly reduced compared with the control group, which had not been subjected to cryopreservation (ANOVA, $p < 0.001$). In the three cryopreserved groups the intact cored sample cartilage discs had the greatest reduction in cell viability. Mincing the cartilage into markedly smaller fragments significantly improved viability (ANOVA, $p < 0.01$).

The results of the cell culture experiment show that chondrocytes from all four groups increased in number over the 28-day period (Fig. 2). However, a common trend in all specimens was a reduction in cell numbers from 5×10^4 up to four days after seeding. This decrease at day four was significantly greater for the cryopreserved specimens (groups B, C, D) than for the freshly isolated unfrozen chondrocytes (group A) (ANOVA, $p < 0.05$). After seven days, cell proliferation was apparent in all four groups with the controls (group A) performing significantly better than the other groups at all time points (ANOVA, $p < 0.01$). At 21 days there had been a significant increase in proliferation between the three cryopreserved groups, with group B (isolated) and group C (minced) increasing at a greater rate than group D (disc) (ANOVA, $p < 0.05$). This difference in cell numbers persisted until day 28, although group B cells decreased in total numbers during this period. Over the

period from day four, when the lowest recorded cell numbers were seen, to day 28, each group expanded by the following mean proportions; group A, 10 times; group B, 18 times; group C, 106 times; and group D, 154 times.

Discussion

This study demonstrates that chondrocytes from cryopreserved human articular cartilage can be isolated and still remain viable and proliferate. However, the viability of cells recovered from minced cartilage specimens was reduced to 30%, compared with the 75% recovery when isolated from specimens of fresh cartilage. These results are consistent with the findings of Jomha et al⁷ who demonstrated a 20% recovery of intact and viable chondrocytes following cryopreservation of intact human cartilage. The results presented here also show that the viability of chondrocytes following cryopreservation is dependent on the size of the tissue sample taken for freezing. By dicing human cartilage tissue to approximately 1.0 mm³ in size, chondrocyte viability was significantly improved after cryopreservation, compared with the intact 5 mm diameter samples of cored cartilage disc. These results are consistent with previous studies using animal cartilage, where it has been noted that chondrocytes in intact cartilage are liable to survive freezing and thawing under well-defined conditions if they are in close proximity (< 50 µm) to a surface.⁸

Poor chondrocyte viability following cryopreservation of whole human cartilage transplantation grafts has been proposed as a limiting factor in their clinical use.^{9,10} The problems faced in cryopreservation of cartilage tissue are not only due to the difficulty of preserving living cells, but also arise from maintaining the properties of the integrated cell/matrix systems, upon which chondrocyte function depends. However, the possibility of using viable cells derived from cryopreserved fragments of cartilage as a resource in autologous chondrocyte implantation has not been fully explored, despite one clinical report of its effectiveness² and being highlighted as an area for further research by a recent NICE review.³ The results from this study show that the viable chondrocytes can be retrieved, cultured and successfully expanded in number to yield a practically useful number of cells. In clinical practice, when undertaking autologous chondrocyte implantation a biopsy is taken at a preliminary arthroscopic procedure and isolated chondrocytes are expanded in number over a six-week period to a total of approximately 20 million cells. The rate of expansion measured in this study demonstrated that it would be possible to use cells isolated from cryopreserved whole cartilage. This is a potentially valuable development when treating a patient with autologous chondrocyte implantation, providing the chondrogenic potential of these cells is still present.

There are some aspects of the method used in this study that require further consideration. The samples of cartilage studied were obtained from patients suffering from severe osteoarthritis. Macroscopically normal cartilage was harvested from the specimens, and has been widely used in the field of cartilage research, owing to the availability of tissue samples. However, the cartilage used in our study may not behave in the same way as that harvested for culture from younger patients during autologous chondrocyte implantation. It is probable that the viability and success of the culture of chondrocytes isolated from non-pathological specimens may be even greater, particularly if younger, non-arthritic specimens are used. This study offers no information regarding the phenotype or function of chondrocytes that have been cryopreserved and expanded in number by culture. Further work is required to determine the effects of cryopreservation on cellular differentiation and the functional abilities of the isolated chondrocytes. Future work will need to investigate the degree of de-differentiation which might be produced, and any changes in the ability to produce collagen II and other specific products of the cartilage matrix.

There are wide variations in the protocols used for isolation and cryopreservation of cells of human articular cartilage.^{5,11-15} We used a constant stepped cooling protocol for cryopreservation with a fast thawing method for all specimens, as has been successfully employed in an animal model.¹⁶ We optimised our protocol after preliminary work examined the effect of enzyme concentration on isolation of cells and cryoprotective agent concentration on the process of freezing and thawing. Further work on these methods may improve viability and the ability of cells to expand in number.

This study has shown that viable chondrocytes, with their proliferative capacity maintained, can be isolated from cryopreserved human articular cartilage. This technique may have a future potential clinical application in autologous chondrocyte implantation.

References

1. Peterson L. Articular cartilage injuries treated with autologous chondrocyte transplantation in the human knee. *Acta Orthop Belg.* 1996; 62(Suppl 1):196–200. [PubMed: 9084569]
2. Minas T. Autologous chondrocyte implantation for focal chondral defects of the knee. *Clin Orthop.* 2001; 391(Suppl):349–61.
3. date last accessed 10 July 2007 Clinical and cost-effectiveness of autologous chondrocyte implantation for cartilage defects in knee joints; technology assessment report. No authors listed <http://www.nice.org.uk>
4. Csonge L, Bravo D, Newman-Gage H, et al. Banking of osteochondral allografts. Part II: preservation of chondrocyte viability during long-term storage. *Cell Tissue Bank.* 2002; 3:161–8. [PubMed: 15256877]
5. Rendal-Vazquez ME, Maneiro-Pampin E, Rodriguez-Cabarcos M, et al. Effect of cryopreservation on human articular chondrocyte viability, proliferation, and collagen expression. *Cryobiology.* 2001; 42:2–10. [PubMed: 11336484]
6. Jomha N, Anoop P, McGann L. Intramatrix events during cryopreservation of porcine articular cartilage using rapid cooling. *J Orthop Res.* 2004; 22:152–7. [PubMed: 14656674]
7. Jomha N, Lavoie G, Muldrew K, Schachar N, McGann L. Cryopreservation of intact human articular cartilage. *J Orthop Res.* 2002; 20:1253–5. [PubMed: 12472237]
8. Muldrew K, Novak K, Studholme C, et al. Transplantation of articular cartilage following a step-cooling cryopreservation protocol. *Cryobiology.* 2001; 43:260–7. [PubMed: 11888219]
9. Lubke C, Sittlinger M, Burmester GR, Paulitschke M. Cryopreservation of artificial cartilage: viability and functional examination after thawing. *Cells Tissues Organs.* 2001; 169:368–76. [PubMed: 11490116]
10. Pegg DE. The current status of tissue cryopreservation. *Cryo Letters.* 2001; 22:105–14. [PubMed: 11788849]
11. Goldberg AJ, Lee DA, Bader DL, Bentley G. Autologous chondrocyte implantation: culture in a TGF-beta-containing medium enhances the re-expression of a chondrocytic phenotype in passaged human chondrocytes in pellet culture. *J Bone Joint Surg [Br].* 2005; 87-B:128–34.
12. Fioravanti A, Cantarini L, Chellini F, et al. Effect of hyaluronic acid (MW 500-730 kDa) on proteoglycan and nitric oxide production in human osteoarthritic chondrocyte cultures exposed to hydrostatic pressure. *Osteoarthritis Cartilage.* 2005; 13:688–96. [PubMed: 15923130]
13. Murphy CL, Polak JM. Control of human articular chondrocyte differentiation by reduced oxygen tension. *J Cell Physiol.* 2004; 199:451–9. [PubMed: 15095292]
14. Fahy GM. The relevance of cryoprotectant “toxicity” to cryobiology. *Cryobiology.* 1986; 23:1–13. [PubMed: 3956226]
15. Almqvist KF, Wang L, Broddez C, Veys EM, Verbruggen G. Biological freezing of human articular chondrocytes. *Osteoarthritis Cartilage.* 2001; 9:341–50. [PubMed: 11399098]
16. Oegema TR, Deloria LB, Fedewa MM, Bischof JC, Lewis JL. A simple cryopreservation method for the maintenance of cell viability and mechanical integrity of a cultured cartilage analog. *Cryobiology.* 2000; 40:370–5. [PubMed: 10924268]

Fig. 1.
Bar chart showing the mean percentage viabilities of chondrocytes following final isolation in the four experimental groups. The error bars represent the 95% confidence intervals.

Fig. 2. Graph showing proliferation of chondrocytes in culture, as indicated by changes in mean cell numbers (95% confidence intervals) with time after seeding for each sample group.