

Brief report

Assessment of strategies to increase chondrocyte viability in cryopreserved human osteochondral allografts: evaluation of the glycosylated hydroquinone, arbutin

S. C. Rosa†, J. Gonçalves†, F. Judas‡, C. Lopes† and A. F. Mendes†*

† Center for Neurosciences and Cell Biology and Faculty of Pharmacy, University of Coimbra, 3004-517 Coimbra, Portugal

‡ Bone and Tissue Bank, Orthopaedics Department, University Hospital of Coimbra, Avenida Bissaya Barreto, Bloco de Celas, 3000-075 Coimbra, Portugal

Summary

Objective: Allogeneic cartilage is used to repair damaged areas of articular cartilage, requiring the presence of living chondrocytes. So far, no preservation method can effectively meet that purpose. Identification of more effective cryoprotective agents (CPAs) can contribute to this goal. The aim of this study was to determine whether the glycosylated hydroquinone, arbutin, alone or in combination with low concentrations of other CPAs, has cryoprotective properties towards human articular cartilage.

Material and methods: Human tibial plateaus were procured from multi-organ donors, with the approval of the Ethics Committee of the University Hospital of Coimbra. The tibial plateaus were treated with or without arbutin (50 or 100 mM), alone or in combination with various concentrations of dimethyl sulfoxide (DMSO) and glycerol, for 0.5–1.5 h/37°C, then frozen at –20°C and 24 h later transferred to a biofreezer at –80°C. Two to 3 months later, thawing was achieved by immersion in cell culture medium at 37°C/1 h. Chondrocyte viability was assessed before and after freeze–thawing using a colorimetric assay based on the cell's metabolic activity and fluorescent dyes to evaluate cell membrane integrity.

Results: Before freezing, chondrocyte metabolic activity was identical in all the conditions tested. After freeze–thawing, the highest activity, corresponding to $34.2 \pm 2.1\%$ of that in the Fresh Control, was achieved in tibial plateaus incubated in 50 mM arbutin for 1 h whereas in those left untreated it was 11.1 ± 4.7 . Addition of DMSO and glycerol to arbutin did not increase chondrocyte viability any further. Fluorescence microscopy confirmed these results and showed that living chondrocytes were mainly restricted to the superficial cartilage layers.

Conclusion: Arbutin seems to be an effective cryoprotective agent for osteochondral allografts with potential benefits over DMSO and glycerol.
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Key words: Arbutin, Chondrocyte viability, Cryopreservation, Human, Tibial plateau.

Introduction

Damage to articular cartilage because of trauma, degenerative and tumour diseases, or infection represents a major clinical problem. Of the existing treatment alternatives, the transplantation of osteochondral grafts is increasingly employed as means of biological resurfacing for articular cartilage defects. Osteochondral grafts, consisting of an articular cartilage layer and the underlying subchondral bone, can either be collected from the patient (autografts)¹ or from multi-organ donors (allografts)^{2,3} which, unlike autografts, allow the treatment of large cartilage defects^{4,5}.

Cryopreserved allografts enable size-matching of the allograft and the recipient, screening of infectious diseases of the donor and quarantine of the allografts, which are mandatory for safe transplantation, and transportation of tissues to distant locations.³ However, the clinical outcome of

cryopreserved vs fresh osteochondral allografts is poor and formation of fibrocartilage, chondromalacia and loss of the cartilage surfaces occur frequently sometime after implantation, leading to a variable graft failure rate of 10–50%.³ The efficacy of the transplanted osteochondral grafts seems to be proportional to their content of living chondrocytes^{6–8}. Since these cells are essential to maintain cartilage matrix integrity⁹, any injury that kills even a small number, can have severe consequences on cartilage integrity and durability.⁸ Cryoprotective agents (CPAs), such as glycerol or dimethyl sulfoxide (DMSO), effectively protect isolated chondrocytes from the damaging effects of freezing.¹⁰ However, freezing of intact articular cartilage, even in the presence of the same CPAs, seriously compromises chondrocyte viability^{7,8,11–14}.

In most studies, a maximum of 20–30% of the cartilage chondrocytes were reported to survive freezing when CPAs, such as DMSO or glycerol, were employed^{11,12,14}, whereas, in their absence, none or a very small number of viable chondrocytes was retrieved^{14,15}. The CPAs currently available are cytotoxic, depending on the duration and temperature of exposure and on the concentrations used. To avoid toxicity, most cryopreservation protocols use low temperatures of exposure

*Address correspondence and reprint requests to: Alexandrina Ferreira Mendes, Center for Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal. Tel: 351-239-480203; Fax: 351-239-480208; E-mail: afmendes@ff.uc.pt

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to the CPAs^{11,12}. However, Carsi and co-workers¹⁵ found that the diffusion of either DMSO or glycerol through human articular cartilage decreased at lower temperatures and with increasing concentrations. Thus, identification of less toxic CPAs should allow longer exposure times at higher temperatures, probably eliciting a more effective cryoprotection. Possible strategies based on this principle are the combination of low concentrations of various CPAs and the identification of new more effective and less toxic CPAs. In this regard, compounds extracted from organisms naturally resistant to drought and cold are being evaluated for cryoprotective properties. One such compound is arbutin (4-hydroxyphenyl- β -D-glucopyranoside, Fig. 1), a glycosylated hydroquinone that has been shown to have a membrane stabilizing effect and to protect isolated cell membranes and liposomes from the damaging effects of both freeze–thawing and drying^{16,17}.

The purpose of this study was to determine whether arbutin has cryoprotective properties towards human articular cartilage. For this and based on the strategies identified above, we evaluated the ability of arbutin, alone and in combination with low concentrations of DMSO and glycerol, to increase the recovery of viable chondrocytes from frozen/thawed whole human tibial plateaus, which represent a good model of the whole bone.

Materials and methods

REAGENTS

The acetomethoxy ester of Calcein, Calcein AM (Cal), was purchased from Molecular Probes (Eugene, OR). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

OSTEOCHONDRAL SAMPLES

As a model of the whole bone, the right and left lateral and medial tibial plateaus, composed of the intact articular cartilage and an underlying bony layer of 0.5–1.5 cm thick, were harvested from 18 multi-organ donors (10 males and 8 females) and processed within 24 h *post-mortem*, according to the standards of the Tissue Bank of the University Hospital of Coimbra (HUC), Portugal, and the Portuguese Transplantation Organization. Donor age ranged from 17 to 53 years old. All the tibial plateaus used appeared macroscopically normal without signs of cartilage damage. All the procedures had the approval of the Ethics Committee of HUC.

CRYOPROTECTION TREATMENTS AND FREEZE–THAWING CONDITIONS

Immediately upon harvesting, the tibial plateaus were incubated at 37°C for 0.5–1.5 h in Ham F-12 nutrient mixture in the presence or absence of 50 or 100 mM arbutin, alone or in combination with DMSO and glycerol in the concentrations indicated in the figures and legends. Then, the tibial plateaus were removed from the cryoprotective solutions, placed in sterile plastic bags, frozen at –20°C/24 h, transferred to a biofreezer at –80°C and kept in those conditions for 2–3 months. Thawing was achieved by immersion in Ham F-12 nutrient mixture at 37°C/1 h.

EVALUATION OF CHONDROCYTE VIABILITY *IN SITU*

Chondrocyte viability *in situ* was assessed before freezing and after thawing. For this, two cartilage cylinders were cut with a 5 mm diameter trephine

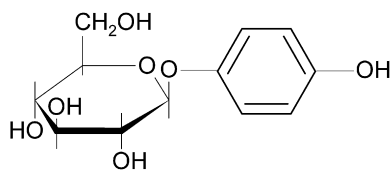


Fig. 1. Chemical structure of arbutin (4-hydroxyphenyl- β -D-glucopyranoside).

from each tibial plateau immediately before freezing and twelve identical cylinders were obtained immediately after thawing. Two methods were used to evaluate chondrocyte viability: (1) an adaptation¹⁸ of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay¹⁹ which is a quantitative colorimetric method based on the reduction of the MTT salt by the mitochondrial enzymes of viable cells; and (2) a cell membrane integrity assay using a fluorescent dye combination consisting of Cal and Propidium Iodide (PI). Cal is a cell membrane-permeable ester that upon hydrolysis by intracellular esterases, emits green fluorescence and becomes cell membrane-impermeable, being retained within cells bearing an intact plasma membrane. PI is cell membrane-impermeable and stains the nuclei of cells with damaged membranes with strong red fluorescence.

The two cylinders collected from each tibial plateau before freezing and ten of those collected after thawing were weighted and incubated in Ham F-12 Nutrient Mixture containing 1 mg/ml MTT, for 1 h at 37°C. The dark blue crystals of formazan produced by reduction of the MTT salt were dissolved in acidified isopropanol and quantified in an automatic plate reader (Tecan/SLT Instruments, Austria) set at a test wavelength of 570 nm and a reference wavelength of 620 nm.

The remaining two cylinders collected after thawing were incubated at 37°C for 30 min in phosphate buffered saline containing 5 μ M Cal and 5 μ g/ml PI. Cryostat sections 30 μ m thick were obtained from each cylinder and viewed under a fluorescence microscope.

DATA ANALYSIS

Where applicable, statistical analysis was performed using one-way analysis of variance with Dunnett's post test. Results were considered significant for $P < 0.05$.

Results

The MTT reduction assay performed in fresh cartilage immediately before freezing [Fig. 2(A)], showed that arbutin did not affect the ability of chondrocytes to reduce the MTT salt, as compared to chondrocytes in cartilage incubated in culture medium alone (Control). These results showed that arbutin was not toxic, nor increased the metabolic activity of chondrocytes *in situ*, under the conditions tested.

After freeze–thawing, the metabolic activity of chondrocytes in tibial plateaus pre-incubated with 50 or 100 mM arbutin for 0.5 or 1 h, was compared to that in the fresh and Post-thawed Controls. As shown in Fig. 2(B), chondrocyte metabolic activity relative to the Fresh Control, which corresponds to the condition of maximal viability, was significantly reduced in all conditions tested. Nevertheless, it was significantly higher in tibial plateaus exposed to arbutin, in any of the conditions tested, than in the Post–thawed Control, which represents the lowest viability, corresponding to $11.1 \pm 4.7\%$ of the metabolic activity of the Fresh Control. The highest activity, corresponding to $34.2 \pm 2.1\%$ of the Fresh Control, was achieved in tibial plateaus incubated in 50 mM arbutin for 1 h, but a shorter incubation period (30 min) elicited almost the same activity ($32.0 \pm 5.4\%$). Increasing arbutin concentration to 100 mM resulted in a slightly lower activity of $28.5 \pm 2.8\%$ relative to the Fresh Control, although the difference was not statistically significant.

Attempting to further enhance *in situ* chondrocyte cryoprotection without significantly increasing direct toxicity, we tested various combinations of low concentrations of DMSO and glycerol with arbutin and extended the incubation period to 1.5 h, hypothesising that diffusion of the CPAs could be improved. The results showed that before freezing, the differences between conditions were not statistically significant, although mean values were slightly variable [Fig. 3(A)]. After freeze–thawing, MTT reduction relative to that obtained in the Fresh Control, was significantly higher with any of the combinations tested than in the Post-thawed Control [Fig. 3(B)], but slightly lower than the highest activity obtained with arbutin alone [Fig. 2(B)].

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