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The effect of additive compounds on glycerol-induced damage to human chondrocytes

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ABSTRACT

High concentrations of cryoprotective agents are required for cryopreservation techniques such as vitrification. Glycerol is a common cryoprotective agent used in cryopreservation protocols but this agent is toxic at high concentrations. This work is an attempt to mitigate the toxic effects of high concentrations of glycerol on intact chondrocytes in human knee articular cartilage from total knee arthroplasty patients by simultaneous exposure to glycerol and a variety of additive compounds. The resulting cell viability in the cartilage samples as measured by membrane integrity staining showed that, in at least one concentration or in combination, all of the tested additive compounds (tetramethylpyrazine, ascorbic acid, chondroitin sulphate, glucosamine sulphate) were able to reduce the deleterious effects of glycerol exposure when examination of membrane integrity took place on a delayed time frame. The use of additive compounds to reduce cryoprotectant toxicity in articular cartilage may help improve cell recovery after cryopreservation.

1. Introduction

Osteoarthritis (OA) is a disease with a substantial personal and economic burden in modern society. In 2014, the World Health Organization (WHO) estimated the 2012 disease burden of OA amounted to a reduction in years of healthy life lost due to disability (disability-adjusted life years - DALY) of over 18 million years worldwide (can be thought of as 18 million individuals having one year of healthy life lost), which has increased since 2000 by over 30% [15]. As there is currently no cure for OA, prevention is paramount. Nonsurgical treatments affect symptoms only while many surgical treatments have limited success and are ineffective when treating larger lesions (>2.5 cm^2) due to the poor healing capability of hyaline (articular) cartilage [10]. This results in few viable options other than synthetic joint replacement for advanced cases of the disease which comes with its own limitations. One surgical treatment that shows promise in treating large defects to prevent the development of OA and maintain joint function is osteochondral allografting, or the transplantation of bone and cartilage from a cadaveric source. The use of cadaveric tissue is possible largely due to the immune-privileged nature of cartilage [19,31], which limits the immune reaction to foreign tissue on transplantation. A significant limitation to osteochondral allografting is that there must be donor cartilage available and that this tissue must be healthy. The structural integrity of cartilage requires healthy cells to continually replace the extracellular matrix, which is responsible for the strength and effectiveness of articular cartilage [7]. This limitation is compounded by the fact that cartilage has a limited capacity to tolerate hypothermic storage (the current storage technique for clinical use), noticeably losing viability after 1–2 weeks [8,56] and dropping to as low as 68% viable after 28 days [18]. These time limitations suggest that an improved preservation method could increase the utility and availability of osteochondral allograft transplantation.

Much of the early focus in the field of cryopreservation was on the preservation of animal spermatozoa [45] and both classical, controlled-rate freezing and vitrification have since been applied with some success in cryopreservation of various cell suspensions,

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but chondrocytes in suspension were not successfully cryopreserved until 1965 [49]. Successful cryopreservation of articular cartilage using classical techniques has proven elusive [22,37,39] primarily due to ice formation and damage through osmotic and mechanical stresses. Vitrification, the formation of a non-crystalline amorphous solid, has proven to be a desirable alternative due to the absence of crystalline ice formation as demonstrated in both porcine [9,21] and, more recently, human applications [3,24]. One drawback to vitrification is the required use of high concentrations of cryoprotective agents (CPAs), such as glycerol, that are cytotoxic. Some methods have been developed to ameliorate this cytotoxicity [6,25,42], including research into transport kinetics for CPAs into tissue [1,2,4,32,36]. Another possibility to reduce CPA toxicity is the use of additive compounds in the cryoprotectant solutions, specifically those compounds that elicit some benefit that is not directly related to the mechanics of vitrification. Glycerol is a CPA that is commonly used in vitrification protocols for various tissues [29,46,53] and was used in our previously successful vitrification of articular cartilage [23,24]; because of its frequent use and some known mechanisms of toxicity, we elected to use glycerol in this initial study.

Examining the role that reactive oxygen species play in joint disorders provides an indication of where additives could be beneficial. It has been previously shown that oxidative stresses are both common and damaging in joint disorders [5], indicating the possible use of a reactive oxygen species scavenging compound as an additive in vitrification treatments tailored to articular cartilage. For the present study, additives were chosen based on antioxidant capacity in consideration with other factors such as previous use with cartilage or pre-approval for medical use. Four additive compounds (chondroitin sulphate (CS), tetramethylpyrazine (TMP), ascorbic acid (AA), and glucosamine sulphate (GlcN)) were evaluated. CS and GlcN are both components of cartilage extracellular matrix and have been used clinically to successfully treat OA [20,38,54]. CS has also been used previously in the vitrification of articular cartilage and has been shown to be beneficial to the cell viability in the rewarmed cartilage [23,24]. AA is a necessary component in collagen synthesis which is in turn a major component of cartilage extracellular matrix. Ascorbic acid treatments in OA settings have also been shown to slow the progression of OA symptoms in humans [35]. Conversely, AA has been seen to increase spontaneous OA symptoms if a concentration that is too high is used (albeit in a guinea pig model) [30]. AA has been investigated in a cryopreservation application, but was found to have no beneficial effects [50]. TMP (clinically Ligustrazine) was traditionally used in Chinese herbal treatments for back and joint pain, and has since been experimentally shown to have anti-inflammatory [28] and anti-apoptotic/reactive oxygen species scavenging capacity [17,26,58].

The purpose of this study was to examine the ability of specific additives to decrease the toxicity of glycerol on human articular chondrocytes. In the present study, TMP, CS, AA, and GlcN were evaluated for their ability to improve cell viability in intact human articular cartilage slices after exposure to a toxic glycerol solution. We hypothesized that the use of these additives would have a beneficial effect on cell viability as determined by membrane integrity measurements.

2. Materials and methods

2.1. Cartilage tissue isolation

Articular cartilage was obtained from human knees undergoing total joint replacement in two local operating rooms (Orthopaedic Surgical Center and Misericordia Hospital, Edmonton, Canada). All tissues were immediately placed in sterile phosphate buffered saline solution (PBS, pH 7.1, Invitrogen, Carlsbad, CA) and stored at 4 °C until use within 1 day of harvest. Although there were no exclusion criteria based on donor characteristics aside from standard research tissue exclusions by the hospitals, information such as height, weight, age, and whether the patient smoked or had undergone cancer or corticosteroid treatment was collected. Ethical approval was obtained from the University of Alberta Ethics Review Board.

The tissues included in the first (immediate evaluation) experiment were taken from 16 adults ranging from 56 to 72 years of age (average 66.0 years, N = 16). In the delayed TMP/CS 48 h analysis experiment, the donors ranged from 52 to 80 years of age (average 64.3 years, N = 10). In the AA/GLcN 48 h analysis experiment, the donors ranged from 52 to 88 years of age (average 64.2 years, N = 10). Ten millimeter full thickness osteochondral dowels were obtained from the discarded tissue of individuals undergoing knee replacements. The 10 mm diameter samples were cored from the best portion of the articular cartilage after visual inspection and grading on the Outerbridge classification system [41], using only "good" tissue graded as a 0 or 1. Articular cartilage was cut from the osteochondral dowels perpendicular to the articular surface into 75 μm thick slices using a vibratome (The Vibratome Company, St. Louis, MO). The slices were immersed in a petri-dish containing PBS at room temperature (22 °C) and used within half an hour of slicing.

2.2. Solution preparation

Experimental solutions were mixed using a base concentration of 1.6 M glycerol (Fisher-Scientific, Ottawa, Ontario) prepared in X-VIVO 10 media (Lonza Inc., Basel, Switzerland), as previous experiments in our lab had shown glycerol to be one of the most toxic cryoprotectants used in our vitrification protocol [6,24]. Eleven experimental additive solutions were used: (i) 0.1 mg/mL chondroitin sulphate (CS; Sigma-Aldrich, St. Louis, Missouri), (ii) 200 μM tetramethylpyrazine (TMP; Sigma-Aldrich, St. Louis, Missouri), (iii) 400 μM TMP, (iv) 200 μM TMP + 0.1 mg/mL CS, (v) 400 μM TMP + 0.1 mg/mL CS, (vi) 500 μM ascorbic acid (AA; L-ascorbic acid 2-phospate sesquimagnesium salt hydrate, Sigma-Aldrich, St. Louis, Missouri), (vii) 1000 μM AA, (viii) 2000 μM AA, (ix) 0.18 mM glucosamine sulphate potassium chloride (GlcN; Medisca Pharmaceutique Inc., St-Laurent, Quebec), (x) 0.36 mM GlcN, and (xi) 0.57 mM GlcN.

In addition to the experimental solutions, there were 3 control solutions: A negative control composed of 8 M glycerol which resulted in 0% cell recovery of a fresh cartilage sample as determined by membrane integrity stains after immersion; a positive control of pure X-VIVO 10; and an unmodified 1.6 M glycerol solution (experimental control which gave ~50% viable after normalization to positive control). Experimental and control solutions were stored in a 4 °C fridge. One millilitre of each solution was placed into a 48-well cell culture plate for use.

2.3. Solution exposure

For each repetition of the experiment, a sufficient number of slices were obtained from the same donor to perform all treatment conditions as well as the positive and negative controls (with duplicate repetitions carried out in any donors with tissue that allowed for the increased number of slices to do so). Thus, there were a minimum of eight slices obtained from each dowel for the first two experiments and a minimum of 11 slices for the third experiment. Slices were exposed to the above solutions for 90 min at room temperature, and then given two 5 min washes in fresh X-VIVO 10. In the TMP/CS groups, slices were evaluated: (i)