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Osmolarity effects on bovine articular chondrocytes during three-dimensional culture in alginate beads

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Summary

Objective: With the development of engineered cartilage, the determination of the appropriate culture conditions is vital in order to maximize extracellular matrix synthesis. As osmolarity could affect the fate of chondrocytes, the purpose of this study was to determine the effects of osmolarity on chondrocytes during relatively long-term culture.

Design: Bovine articular chondrocytes were cultured in alginate beads in a biocarbonate free system at 280, 380 and 550 mOsm at pH 7.4 for up to 12 days, respectively. Cell volume, intracellular pH (pH_i), cell number, glucosaminoglycan (GAG) and collagen retention were measured at day 5 and 12. Cell viability and volume were monitored over the 12 days of culture.

Results: By day 5 and 12, compared to the cell volume at 380 mOsm, around 20% ($P < 0.01$) swelling and 15% ($P < 0.05$) shrinkage were observed when the cells were cultured at 280 and 550 mOsm. The pH_i over the 12 days of culture varied with osmolarity of the culture medium. In comparison with fresh cells, pH_i became slightly more acidic by 0.15 pH units at 280 mOsm at day 5. However, by day 12, an alkalization of pH_i, by 0.2 pH units, was noted. A higher proliferation rate was seen at 280 mOsm than at other osmolarities while less GAG was produced.

Conclusions: Chronic exposure to anisotonic conditions results in cell swelling at 280 mOsm and shrinkage at 550 mOsm. The osmolarity of 280 mOsm appears to encourage proliferation of chondrocytes, but inhibits matrix production.

Key words: Articular chondrocytes, Osmolarity, Cell volume, Intracellular pH, Matrix production.

Introduction

Chondrocytes, as the only cell type in articular cartilage, are critical for the development of engineered cartilage. In native articular cartilage, chondrocytes are sparsely embedded in a hydrated extracellular matrix containing mainly collagens and proteoglycans (PGs)[1]. The PGs through their regulation of the extracellular ionic environment impart a high extracellular osmolarity and hence swelling pressure to the cartilage matrix. The extracellular osmolarity is determined mainly by the local PG concentration in different cartilage zones and varies from around 350 mOsm in the surface zone of human articular cartilage to around 450 mOsm in the mid-zone[2,3]. Cartilage osmolarity alters during pathological changes to the matrix as a consequence of changes in aggrecan concentration. Hence it might be expected that osmolarity would affect the cartilage functions, which would be important to cartilage tissue engineering.

To date cell responses under exposure to anisotonic conditions, in which chondrocytes are acutely exposed to media with osmolarity outside the physiological range of 350–450 mOsm, have been extensively studied[4–7]. Chondrocytes, like many other types of cells, have the capacity to limit cell volume changes during exposure to hypotonic or hypertonic conditions, either by using regulatory volume decrease (RVD), which is achieved by the activation of ion transport pathways and non-selective "osmolyte channels"[5–7], or by means of regulated volume increase (RVI), which is mediated mainly by activation of the Na/K/2Cl cotransporter[8]. Through this regulation chondrocytes maintain optimal volume and hence also limit any changes in cell metabolism and biosynthesis[3] arising from the signaling pathways induced following exposure to acute osmotic challenge.

However, the responses of chondrocytes to chronic exposure to changes in extracellular osmolarity, which may provide information on selection of optimal culture conditions for the development of engineered cartilage, are still not clear. Hence, in this study, the effects of chronic exposure to controlled extracellular osmolarities on chondrocyte behavior were investigated. Chondrocytes were seeded into 3D alginate beads, and then cultured at three different osmolarities, 280, 380 and 550 mOsm for 12 days. The cell volume, intracellular pH, cell number and extracellular matrix retention were monitored over the 12 days of culture.

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Materials and method

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Poole, UK.

ISOLATION OF CHONDROCYTES

Chondrocytes were isolated by an enzyme digestion method as previously described[9]. Articular cartilage was cut from the metacarpophalangeal joint of bovine feet obtained from the local abattoir and stored at 4°C until used (within 48 h). The cartilage was then dissected under sterile conditions and incubated in Dulbecco's Modified Eagle's Medium (DMEM) with 25 mM N-2-Hydroxyethyl piperazine-N′-2-ethanesulfonic acid (HEPES) plus collagenase type I (1 mg/ml) and antibiotic 1% (v/v) penicillin (10,000 units/ml) and streptomycin (10 mg/ml), and amphotericin (250 μg/ml), (Life Technologies, Paisley, UK) at 37°C on rollers for 18 h. The medium was adjusted to 380 mOsm using 5 M NaCl solution to limit cell swelling during digestion. After isolation, cell number and cell viability were assayed using a trypan blue (4% wt/wt) exclusion test on a hemacytometer. Only isolations where cell viability was greater than 96% were used for further experiments.

CHONDROCYTE CULTURE IN ALGINATE BEADS

Chondrocytes were then encapsulated into alginate beads following the method of Guo *et al*[10]. Briefly, after centrifuging, the cell pellet was resuspended in a sterilized 0.9% NaCl solution containing 1.2% low viscosity alginate (Merck, UK) at a cell density of 4×10^6cells/ml. The cell suspension was slowly pressed through a 22 gauge needle into a sterilized 102 mM $CaCl_2$ solution. The alginate beads, approximately in diameter of 1 mm, were polymerized for 10 min, washed twice with the sterile 0.9% NaCl solution, followed by two washes in the culture medium. The alginate beads containing chondrocytes were then transferred to 24-well microplates.

Three culture media at different osmolarities were tested in this study. In all cases the culture medium was DMEM buffered by HEPES (25 mM), and supplemented with 50 μg/ml L-ascorbic acid, 10% fetal bovine serum and 2% (v/v) antibiotic. The osmolarity of the culture medium was adjusted using NaCl and KCl salt to 280, 380 and 550 mM, maintaining Na/K ratios equal to those in DMEM. The osmolarity of the culture media was measured using a freezing point osmometer 030 (Geotech GmbH, Germany). The pH of all culture media was adjusted to pH 7.4 using 5 N NaOH.

The alginate beads were cultured in 24-well microplates in a humidified air incubator at 37°C for up to 12 days. The beads (3 beads/well) where cultured in 2 ml culture medium at one of three different osmolarities (280, 380 and 550 mOsm). The culture medium was replaced every 2–3 days. The pH of the culture medium was measured before each replacement. The spent culture media were stored at −20°C until assayed. Cultured beads were harvested at the end of day 5 and day 12 for biochemical evaluation and cell volume measurement.

CELL VOLUME MEASUREMENT

Chondrocyte volume in alginate beads was monitored non-invasively using a multiphoton microscope (MPM). *In situ* chondrocyte volume was measured at day 5 and at day 12 at for chondrocytes cultured at each of 280, 380 and 550 mOsm. Before measurement, samples from culture at 280, 380 and 550 mOsm were stained with Live/Dead (Calcein-AM/Ethidium homodimer-1, (EB) Molecular Probes, UK) at 1 μM for 20 min at 37°C at the corresponding osmolarity. Live cells show green color, and dead cells are red. The boundary of the chondrocytes was determined by staining with calcein. Then the samples were washed three times using culture medium with the osmolarity of 280, 380 and 550 mOsm, respectively, and then kept the samples in an observation slide, in which the samples were surrounded by the corresponding culture medium.

Here a modified femtosecond near infrared two/multiphoton laser scanning system[11] was used for the non-invasive 3D imaging of specimens as recently described and detailed by Tirlapur *et al*[12]. Sub-femtolitre excitation beam focusing was achieved using a high-numerical-aperture (NA, 1.3) 40× water immersion objective (Nikon), with a working distance of ca 1 mm. Stepper motor control of the objective lens focus enabled scanning along the optical *z*-axis with a minimum step size of 1.5 μm. In the multiphoton dedicated system provided by Bio-Rad, a single 670 nm ultraviolet optimised long-pass dichroic mirror (Chroma Tech. Corp, Vermont, USA), placed in the excitation path within the infinity focus of the microscope head directed the fluorescence emission signal in the ultraviolet (UV) to visible wavelength range towards non-descanned bi-alkaline and multi-alkaline Photomultiplier Tubes. Mean laser powers were electronically regulated *via* the pockels cell of the Body Cooling Unit (BCU) and were recorded using a Fieldmaster (FM) power meter (Coherent, Ely, UK) at the back aperture of the objective lens. Multiphoton excitation of the chondrocytes associated fluorescence of calcein and EB required less than 1 mW of mean laser power.

Images were loaded either into Imaris 4.2 (Bitplane Ag, Zurich, Switzerland) or the LaserSharpe (Zeiss, Jena, Germany) software for processing and analysis[13]. The Imaris software suite offers a range of highly advanced three-dimensional (3D) image processing capabilities including volume rendering, orthogonal plane projections, and statistical analysis of 3D surface-rendered objects. Each of these techniques was used to evaluate the chondrocyte volume in the 3D constructs.

INTRACELLULAR pH (pH_i) MEASUREMENT

Three measuring solutions were made using NaCl and KCl with 25 mM HEPES at 280, 380 and 550 mOsm, and the pH was adjusted to pH 7.4 using 5 N NaOH. A fluorimetric technique using the pH-sensitive dye, 2′, 7′-bis (carboxyethyl)-5(6)-carboxyfluorescein (BCECF), which has been successfully applied to the determination of pH_i of chondrocytes[14,15], was adopted to determine the pH_i of chondrocytes. The cells released from alginate beads were incubated in the solution with 10 μM (BCECF-AM) (Calbiochem, UK) at the corresponding osmolarity for up to 30 min, washed twice, then resuspended in the relevant solution, then transferred to a cuvette for fluorimetric measurement (F-2500 spectrophotometer, with magnetic stirrer and thermostat, Hitachi, Japan). The ratio of the intensity of fluorescent emission at 535 nm with two excitation wavelengths at 439 and 490 nm, $R = I_{490}/I_{439}$, is used to measure the pH_i.

The *R* value was converted to pH using the well-established nigericin-high K^+ calibration method[16]. In calibration, chondrocytes were loaded with BCECF in HEPES buffer with KCl (150 mM), supplemented with the K^+/H^+ exchanging ionophore nigericin (3 μM) which causes a rapid equilibration of the extracellular and pH_i by the exchange of intracellular K^+ and extracellular H^+[18]. The pH of solution was adjusted to three different pH values in the range of 6.3–8 using concentrated potassium hydroxide (KOH). The measured *R* values were then plotted against pH. A link between *R* and pH was established. All the experiments were carried out at 37°C.

CELL NUMBER

The number of chondrocytes in the beads was determined by deoxyribonucleic acid (DNA) measurement using Hoechst 33,258 analysis[17]. The alginate beads were digested with 125 μg/ml papain solution (55 mM sodium citrate and 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraacetic acid (EDTA) in 0.9% NaCl) at 60°C overnight. Cell concentration was determined from the DNA content of the digest, using a value of 7.7 pg DNA/chondrocyte. With calf thymus DNA as a standard, DNA concentration was measured using a microplate reader (Genois, Tecan, UK) at excitation of 360 nm and emission of 430 nm.

MEASUREMENT OF GLUCOSAMINOGLYCAN (GAG) AND COLLAGEN SYNTHESIS

At day 5 and day 12, the beads were frozen and digested. Before assay, the alginate beads were first solubilized in 55 mM sodium citrate in 0.9% NaCl for 15 min, and then digested with 125 μg/ml papain solution. With chondroitin sulphate A sodium salt as the standard, total content of sulfated glycosaminoglycans (sGAG) in spent media was determined using dimethylmethylene blue (DMB) assay[18]. GAG in alginate beads was determined spectrophotometrically at 600 nm (Genois, Tecan, UK) using the modified DMB method[19], with the pH of DMB dye solution at pH 2.3 to suppress the background caused by alginate. Total collagen concentration in alginate beads was determined from the hydroxyproline concentration[20] after hydrolysis with 6 N HCL at 115°C for 18 h. Hydroxyproline was measured at the wavelength of 540 nm after reaction with chloramine-T and p-dimethylaminobenzaldehyde.

STATISTICAL ANALYSIS

At each condition, experiments were carried out in triplicate each time. All experiments were repeated at least three times, using the cells from the different animals. Unless stated otherwise, data are presented as the mean ± the standard error of the mean (S.E.M) of at least three separate independent experiments ($n \geq 3$). To determine the effects of osmolarity on chondrocyte responses (cell volume, intracellular pH, DNA content, matrix production) during long-term culture, statistical comparisons were made using an unpaired, two-tailed Student's *t*-test with a confidence level of 95%.

Results

OSMOLARITY AND pH OF CULTURES

The experiments were carried out at three different osmolarities, 285 ± 15, 390 ± 8 and 557 ± 12 mOsm, respectively