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 bicarbonate free system at 280, 380 and 550 mOsm at pH 7.4 for up to 12 days, respectively. Cell volume, intracellular pH (pHi), 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, over the 12 days of culture varied with osmolarity of the culture medium. In comparison with fresh cells, pHi became slightly more acidic by 0.15 pH units at 280 mOsm at day 5. However, by day 12, an alkalization of pHi, 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.

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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). 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. 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. 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”, or by means of regulated volume increase (RVI), which is mediated mainly by activation of the Na/K/2Cl co-transporter. Through this regulation chondrocytes maintain optimal volume and hence also limit any changes in cell metabolism and biosynthesis 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.

ISOULATION OF CHONDROCYTES

Chondrocytes were isolated by an enzyme digestion method as previously described. 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 (100 mg/ml) penicillin (10 mg/ml) and streptomycin (10 mg/ml), and amphotericin (250 μg/ml), (Life Technologoies, 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/vt) 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. Brieﬁly, after centrifuging, the cell pellet was resuspended in a sterile NaCl solution at a concentration of 1.2% low viscosity alginate (Merck, UK) at a cell density of 4 × 10⁶ cells/ml. The cell suspension was then slowly pressed through a 22 gauge needle into a sterilized 102 mM CaCl₂ solution. The alginate beads, approximately in diameter of 1 mm, were polymerized for 10 min, washed twice with the sterile 0.9% NaCl solution, and 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. 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% antibiotic. The osmolarity of the culture medium was adjusted 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 of chondrocytes, was adopted to determine the pH of chondrocytes. The 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 reus- pondered in the relevant solution, then transferred to a cuvette for ﬂuorimetric measurement (F-2500 spectrophotometer, with magnetic stirrer and thermo- stat, Hitachi, Japan). The ratio of the intensity of ﬂuorescent emission at 535 nm with two excitation wavelengths at 439 and 490 nm, R = I₄₉₀/I₄₃₉, is used to measure the pH.

The R value was converted to pH using the well-established nigericin-high K⁺ calibration method. In calibration, chondrocytes were loaded with BCECF in HEPES buffer with KCl (150 mM), supplemented with the K⁺ exchanging ionophore nigericin (3 μM) which causes a rapid equilibration of the extracellular and pH by the exchange of intracellular K⁺ and extracellular H⁺. 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 line 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 deoxyribonu- clease acid (DNA) measurement using Hoechst 33,258 analysis. The algi- nate beads were digested with 125 μg/ml papain solution (55 mM sodium citrate and 5 mM cysteine hydrochloride and 5 mM ethylenediaminetetraae- toc acid (EDTA) in 0.9% NaCl) at 60°C overnight. Cell concentration was de- termined from the DNA content (3 μM), using a value of 7.7 pg DNA per chondrocyte. With calf thymus DNA as a standard, DNA concentration was measured using a microplate reader (Genos, 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 collagen and glycosaminoglycans (sGAG) in spent media was measured using dimethylthylene blue (DBM) assay. GAG in alginate beads was determined spectrophotometrically in 600 μM (Genos, Tecan, UK) using the modiﬁed DBM method, with the pH of DMEM 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 after hydrolysis with 6 N HCl at 115°C for 18 h. Hydroxyproline was measured at the wavelength of 540 nm after reaction with chloroamine-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 in- dependent experiments (n ≥ 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 CHONDROCYTE CULTURES

The experiments were carried out at three different osmolarities, 285 ± 15, 390 ± 8 and 557 ± 12 mOsm, respectively.
When chondrocytes were cultured in 1.2% alginate beads with these different culture media, the pH of culture medium was measured prior to each change. During 12 days of culture, the pH was kept at 7.31 ± 0.03, 7.30 ± 0.05, and 7.34 ± 0.06, respectively (n = 4).

CELL VOLUME MEASUREMENT

Figure 1 shows chondrocyte morphology in alginate beads in the outer region and in the deep zones; results are shown from the surface to 150 µm depth, and from 300 µm to 450 µm depth, in alginate beads at day 5 and 12 of culture. Chondrocytes remained the round shape in both regions for the whole culture period.

The cell volume in the surface region and in the deep zones was measured at day 5 and 12. By day 5, the cell volume at 280 mOsm was greater by 21.05 ± 3.86% (P = 0.021, n = 4) in the surface region and 20.05 ± 3.86% (P = 0.001, n = 4) in the deep zones than that at 380 mOsm, and lower by 80.38 ± 6.95% (P = 0.005, n = 4) in the surface region and 89.90 ± 4.53% (P = 0.045, n = 4) in the deeper zone at 550 mOsm than that at 380 mOsm. By day 5 in culture, the cell volume in both regions in alginate beads stabilized. No significant increase or decrease in the cell volume at day 12 was observed compared to the cell volume at day 5, 28.21 ± 5.58% (P = 0.005, n = 3) swelling in the surface region and 25.87 ± 4.27% (P = 0.033, n = 3) swelling in the deep zone than that at 380 mOsm, and 13.13 ± 1.02% (P = 0.009, n = 3) in the surface region and 13.29 ± 5.84% (P = 0.037, n = 3) in the deep zone (Fig. 2).

pH MEASUREMENT

At the end of 5 days of culture, pH of chondrocytes cultured at 280 mOsm was 0.2 pH units lower than that at 380 mOsm (P = 0.007, n = 4), whereas pH of chondrocytes cultured at 550 mOsm was around 0.15 units lower than that at 380 mOsm (P = 0.037, n = 3). By day 12, an acidification of pH, approximately 0.1 units, was observed at 380 mOsm, but this is not statistically significant. In contrast, at 280 mOsm, a pronounced increase in pH, 0.3 units, was achieved.

CELL NUMBER

The DNA content was normalized to the ratio of DNA content at the initial cell seeding number in 1.2% alginate beads (Fig. 4). At the end of 5 days of culture, cell number fell under hypertonic condition, 550 mOsm (P = 0.03, n = 4). By day 12, a noticeable increase in the cell content was observed at 280 mOsm. At 550 mOsm, the cell content returned to the initial cell density.

BIOSYNTHESIS ACTIVITY

Figure 5 shows GAG retention and collagen accumulation in the beads after 5 and 12 days of culture. GAG retention in alginate beads increased with culture time from day 5 to day 12. When GAG retention was normalized to the initial cell seeding density [Fig. 5(A)], GAG retention was the greatest at 380 mOsm, and the lowest at 280 mOsm, but not at levels of significance. However, when normalized to the cell number at day 5 and day 12 of culture, significantly low GAG retention occurred at 280 mOsm compared to that at 380 and 550 mOsm [Fig. 5(B)] by day 12 (P = 0.011, n = 4). In Fig. 5(C), hydroxyproline accumulation in alginate beads was normalized to the cell number at day 5 and day 12. No significant difference in hydroxyproline accumulation was observed for the tested conditions.

Discussion

For successful development of engineered cartilage, it is important to determine which culture conditions are most favourable for extracellular matrix synthesis. Here we investigated the effects of chronic exposure (up to 12 days) to
a range of different osmolarities on the cell volume and cell functions of bovine articular chondrocytes cultured in 3D alginate beads. During 12 days of culture, alginate beads maintained stable, a stable extracellular pH was maintained closed to pH 7.3 under all culture conditions by using a large volume of culture medium, thus avoiding possible effects caused by fluctuation in extracellular pH, which can affect matrix synthesis by chondrocytes. The data obtained in this study show that extracellular osmotic environment regulates cell volume and affects cellular functions such as cell proliferation and matrix production. It should be pointed out that due to the low number of observations ($n = 3$ or 4), it is difficult to detect whether the assumption of Gaussian distribution which student's $t$-test is based is valid.

We found that the cell volume of chondrocytes, cultured for up to 12 days in alginate beads, varied with the extracellular osmolarity. The volume of cultured chondrocytes was highest at 280 mOsm, and lowest at 550 mOsm at both the interface and deep zones of alginate beads after 12 days of culture (Fig. 2) Cell volumes had stabilized within 5 days of culture; cell volumes at day 5 were similar to those at day 12 under all conditions tested implying adaptation to prevailing osmolarity over this culture period (Fig. 2). This adaptation possibly involves resynthesizing receptors regulating cell volume and pericellular matrix assembly which could contribute to regulation of cell volume.

The response of chondrocytes cultured in 3D alginate to the prevailing osmolarity is however different from that in native cartilage. In ex vivo cartilage, unlike the results we found in culture, cells volume is smallest where the osmolarity is lowest, i.e., in the surface zone, while cell volume is significantly greater at the interface and in the deep zone where the osmolarity is considerably greater. It thus seems that osmolarity alone does not regulate chondrocyte volume in situ as zonal variation in composition and fixed charge density (FCD) in native tissue may play a role in cell volume, which is affected by cell–matrix interactions.

In addition, the difference of chondrocyte behavior between ex vivo cartilage and in 3D alginate could be caused by enzyme treatments during isolation. Some signaling pathways of chondrocytes could be compromised by the extraction of cells from extracellular matrix even though the pathways mediating acute regulatory cell volume responses appear intact. Matrix accumulation appears to influence response of chondrocytes to osmotic stimuli; matrix composition could thus be important in regulating cell volume through other factors in addition to those regulating extracellular osmolarity. Differences in cell population between surface and deep zones could also be important; for cells from each zone could have intrinsically different cell volumes and thus show differential responses to the same extracellular osmolarity. It should be noted however that although there are distinct differences in volumes of surface and deep zone chondrocytes on isolation, these differences in size are reported to disappear in culture. In our culture system, the chondrocytes were not from the single zone of cartilage but were a mixed population from the whole depth of cartilage.

The properties of scaffolds which have the capacity to retain the products produced by cells plays a vital role in tissue engineering. It has been demonstrated that scaffold properties affect cell behavior such as cell proliferation, cell volume, matrix synthesis and retention. In neutrally charged agarose, increase in cell volume with culture time has been observed during 40 days of culture, yet, in
alginate, cell volume became adapted to the prevailing osmolarity during 5 days of culture under all conditions tested (Fig. 2). These contrasting responses in cell volume change with culture time may be caused by nature of scaffolds used. The properties of negatively charged alginate gel depend on their composition, the ratio of β-D-mannuronic acid (M) and α-L-guluronic acid (G)\(^28\). Mechanical stiffness of alginate decreases with the increase in concentration of Na\(^+\) due to dissolution through ion exchange of calcium even under physiological conditions\(^29\). Although it has been demonstrated that low mechanical stiffness can promote cell proliferation\(^29\), we did not observe a high cell proliferation rate when chondrocytes in alginate were exposed to an osmolality of 550 mOsm achieved by the addition of NaCl, even the latter would result in weaker strength of the gel. It hence seems that mechanical stiffness alone does not regulate cell responses in the range tested in this study.

It is interesting to note that the cell volume change under chronic exposure to anisotonic conditions does not follow the pattern of the cells exposed to acute osmotic challenge. When the chondrocytes are exposed to acute anisotonic conditions, there is a rapid change in cell volume. Na\(^+\), K\(^+\) or Cl\(^-\) channels open and there is immediate activation of the volume-sensitive organic osmolyte channel of taurine or betaine\(^30\)–\(^33\) followed by enhancement of gene expression of osmolyte transporters\(^34\). This results in recovery of original cell volume through accumulation or loss of betaine or taurine\(^32\)–\(^35\). If the extracellular osmolarity remains anisotonic, gene expression of betaine or taurine transporters fall steeply to a new lower steady-state level\(^32\). This decrease in expression results in down regulation of osmolyte influx or efflux compared to that under acute anisotonic challenge\(^32\). Thus the mechanism for cell volume regulation in response to chronic anisotonic conditions differs from that in response to acute osmotic shock and could explain why cell volume though initially restored to its original level then alters in response to prevailing osmolarity. It should be noted that with adaption of the cells to their new prevailing osmolarity the final intracellular concentration of Na\(^+\), K\(^+\) and Cl\(^-\) is different from the initial value before application of osmotic shock. This alteration in intracellular ionic composition could possibly explain some of the changes in behavior noted here (Figs. 3–5). However, in order to distinguish the effects of the cell volume regulation itself vs cell volume changes controlled by the physicochemical surroundings, further studies on cell volume regulation,
perhaps using inhibitors to isolate individual effect, are required.

Intracellular pH, determined by the balance between the rates of acid loading and acid extrusion from cells and control by the intracellular buffering power, plays an important role in chondrocyte metabolism.37 Chondrocytes maintain steady-state pH, mainly through the Na\(^+\)\(-\)H\(^+\) exchanger (NHE)38. It has been reported that pH, is altered by acute exposure to anisotonic shock15,39. However, the pattern of pH, alteration of chondrocytes in response to chronic osmotic challenge differs from the cells in response to acute osmotic stress. Exposure to acute anisotonic conditions results in an alkalization of pH\(_{i}\) by NHE. In contrast, in the current study, in response to chronic hypotonic exposure, the pH\(_{i}\) of chondrocytes was more acidic, by 0.15 pH units, than in the freshly isolated cells by day 5, whereas, by day 12, became more alkaline, by 0.2 pH units, than the freshly isolated chondrocytes (Fig. 3). However, the acidification may happen at an earlier stage as we only measured the pH\(_{i}\) at day 5. We cannot explain what causes pH\(_{i}\) of chondrocytes to shift from acidic to alkaline between day 5 and day 12. It might be caused by the changes in parameters such as intracellular ion strength or concentration of intracellular ions under chronic osmotic stress. In addition, the release of chondrocytes from alginate during pH measurement may cause change in pH\(_{i}\). Hence, a further investigation is underway.

Cell proliferation can be affected by alteration of pH\(_{i}\) and osmotic conditions.40-43 Indeed cellular alkalization is believed to enhance cell proliferation36. Conversely, lowering pH\(_{i}\) in many cell types inhibits cell proliferation15. In addition, cell proliferation under hypotonic conditions is controversial. Exposure to hypotonic condition results in slow growth rate for mouse embryonic stem cells,36 but induces an increase in cell proliferation of keratinocytes.44 Hence the response of cell proliferation to a hypotonic culture environment may be cell-type-related. In the current study, by day 12, pH\(_{i}\) was more alkaline, (by 0.2 pH units) at 280 mOsm, than that at the other two osmolarities and more cells were detected at 280 mOsm implying a high proliferation rate. On the other hand, cell proliferation rate was inhibited by the slight acidification of pH\(_{i}\) at 550 mOsm. Hence, in cartilage tissue engineering, cell proliferation could be enhanced by hypotonic culture conditions.

In chondrocytes, matrix production is very sensitive to osmotic stress.13 In terms of GAG retention based on the initial cell density, no significant difference in GAG retention was seen with change in osmolarity, possibly masked by the large standard deviations of our results. However, normalized to the cell number at day 5 and at day 12, there was a dramatic decrease in matrix retention in chronic hypotonic culture (Fig. 5). There would be a loss in GAG during 12 days of chondrocyte culture in alginate beads, as demonstrated in the previous study under physiological culture environment45. Even when GAG loss into the media was considered (data not shown), GAG production in chronic anisotonic condition was the lowest at 550 mOsm. This result is consistent with a previous report46, in which \(^3\)H-sulphate incorporation was suppressed by acute hypertonic challenge. However, it should be noted that biosynthesis by chondrocytes may be different in a bicarbonate-buffered system.

In conclusion, the prevailing extracellular osmolarity of the culture medium around the chondrocytes exerts significant effects on cell size, intracellular pH, cell proliferation and matrix production. Hence in cartilage engineering, a precisely controlled culture environment may be critical to grow functional tissues.

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