

Osteoarthritis and Cartilage



Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes

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ARTICLE INFO

Article history:

Received 17 December 2009

Accepted 14 July 2010

Keywords:

Cartilage repair
Agarose hydrogel
Infrapatellar fat pad
MSCs
Chondrocytes
Functional properties
Oxygen tension

SUMMARY

Background: For current tissue engineering or regenerative medicine strategies, chondrocyte (CC)- or mesenchymal stem cell (MSC)-seeded constructs are typically cultured in normoxic conditions (20% oxygen). However, within the knee joint capsule a lower oxygen tension exists.

Objective: The objective of this study was to investigate how CCs and infrapatellar fat pad derived MSCs will respond to a low oxygen (5%) environment in 3D agarose culture. Our hypothesis was that culture in a low oxygen environment (5%) will enhance the functional properties of cartilaginous tissues engineered using both cell sources.

Experimental design: Cell-encapsulated agarose hydrogel constructs (seeded with CCs or infrapatellar fat pad (IFP) derived MSCs) were prepared and cultured in a chemically defined serum-free medium in the presence (CCs and MSCs) or absence (CCs only) of transforming growth factor-beta3 (TGF-β3) in normoxic (20%) or low oxygen (5%) conditions for 42 days. Constructs were assessed at days 0, 21 and 42 in terms of mechanical properties, biochemical content and histologically.

Results: Low oxygen tension (5%) was observed to promote extracellular matrix (ECM) production by CCs cultured in the absence of TGF-β3, but was inhibitory in the presence of TGF-β3. In contrast, a low oxygen tension enhanced chondrogenesis of IFP constructs in the presence of TGF-β3, leading to superior mechanical functionality compared to CCs cultured in identical conditions.

Conclusions: Extrapolating the results of this study to the *in vivo* setting, it would appear that joint fat pad derived MSCs may possess a superior potential to generate a functional repair tissue in low oxygen tensions. However, in the context of *in vitro* cartilage tissue engineering, CCs maintained in normoxic conditions in the presence of TGF-β3 generate the most mechanically functional tissue.

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Introduction

There has been increased interest in utilising mesenchymal stem cells (MSCs) for cell-based cartilage repair therapies. This has been partially motivated by the well-reported loss of phenotype during monolayer expansion of isolated chondrocytes (CC)^{1,2}, as well as age and disease-related loss of chondrogenic potential^{3,4}. MSCs derived from the bone marrow have attracted significant attention for cartilage therapy development^{5–10}. More recently, it has also been demonstrated that non-cartilaginous knee joint tissues such as the infrapatellar fat pad (IFP)^{11–16} and

synovium^{17–22} possess significant chondrogenic potential and perhaps provide a more readily available and clinically feasible source of chondroprogenitor cells. Incorporating such cells into scaffolds or hydrogels represents a promising strategy for engineering cartilaginous grafts. Hydrogels such as agarose are commonly used for cartilage tissue engineering applications^{6,23–32}. Agarose is a polysaccharide extracted from marine red algae, which has been shown to support the chondrogenic phenotype³³, the elaboration and synthesis of pericellular³⁴ and cartilage extracellular matrix (ECM)^{35–37} by CCs, as well as facilitating chondrogenesis of MSCs^{23,38}. This is typically achieved by stimulating encapsulated cells with growth factors such as transforming growth factor-β3 (TGF-β3)^{6,16,39–42}. In addition, agarose has previously been used in animal model studies for cartilage defect repair^{43,44}. Clinical studies have also been undertaken investigating agarose–alginate hydrogels seeded with CCs for human cartilage

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defect repair^{45,46}. Identifying the environmental conditions that promote chondrogenesis within such scaffolds and hydrogels represents a key challenge in the field of cartilage tissue engineering.

Oxygen tension would appear to play a key role in regulating chondrogenesis during tissue development and regeneration. There is a strong relationship between oxygen concentrations and tissue differentiation during limb development⁴⁷ and during *in vivo* fracture repair with low oxygen tension favouring the formation of bone through endochondral ossification^{48–50}. In cartilage explant models, low oxygen tension has been shown to promote cartilage specific ECM production^{51,52}, with oxygen tensions of 5% shown to significantly increase proteoglycan and collagen synthesis compared to culturing at 20% or at anoxic conditions (1%)⁵³. In addition, when combined with intermittent dynamic compression, oxygen tension has been shown to have a significant effect on the induction of inflammatory mediators in cartilage explants such as nitric oxide and prostaglandin E2⁵⁴.

From a regenerative medicine or tissue engineering perspective, oxygen tensions and oxygen gradients would also appear to play a key role in regulating the phenotype and biosynthetic activity of cells intended for therapeutic applications⁵⁵. It has been demonstrated that a combination of alginate encapsulation and reduced oxygen tension (5%) can restore the chondrogenic phenotype of monolayer-expanded dedifferentiated cells^{56,57}. Similar results have been observed in pellet culture studies for dedifferentiated nasal CCs⁵⁸. Follow-up studies have shown that hypoxia promotes re-establishment of the chondrogenic phenotype through HIF-2 α -mediated SOX9 induction of key cartilage genes⁵⁹. A number of other studies have also investigated how the oxygen microenvironment influences CC proteoglycan and type II collagen synthesis^{60–64}. Bovine articular CCs cultured under anoxic conditions (<0.1%) in alginate beads produced less matrix than for higher oxygen tensions (5, 10 and 20%), with highest expression levels observed when cultured in 5–10% oxygen⁶³. Sustained hypoxia (2%) has also been shown to enhance articular CC matrix synthesis and viability in three-dimensional alginate culture with increases in proteoglycan synthesis after only 1 day of exposure as measured by 35S-sulfate incorporation⁶⁵. In contrast, other studies have demonstrated enhanced cartilage matrix production at higher oxygen tensions⁶⁶.

In addition to regulating the phenotype and biosynthetic activity of differentiated CCs, hypoxia also appears to be a key regulator of MSC chondrogenesis. Hypoxia has been shown to enhance chondrogenesis of bone marrow derived MSCs *via* a HIF-1 α -dependent mechanism^{67,68}, and also influences chondrogenesis of human embryonic stem cells⁶⁹. Bone marrow derived MSCs cultured in reduced oxygen tension produced more bone and cartilage when loaded onto ceramic cubes and implanted subcutaneously than do those cultured at control oxygen levels⁷⁰. Low oxygen tension has also been shown to significantly inhibit the proliferation of adipose tissue derived stem cells embedded in alginate beads, but with increased levels of cartilage matrix synthesis, suggesting oxygen tension regulates the proliferation and metabolism of stem cells as they undergo chondrogenesis⁷¹. Oxygen tension has also been shown to regulate type X collagen levels in human adipose-derived adult stem cells⁷². However other studies have suggested that hypoxia (2%) strongly inhibits *in vitro* chondrogenesis and osteogenesis of adipose-derived stem cells⁷³.

There is therefore some uncertainty in the literature as to how CCs and MSCs undergoing chondrogenesis will respond to altered oxygen conditions, and what role this key regulatory factor plays in cartilage tissue engineering⁵⁵. The objective of this study was to investigate how CCs and IFP derived MSCs would respond to a low oxygen environment in 3D agarose hydrogel culture. To address

this question, cell-encapsulated agarose hydrogel constructs were cultured in a chemically defined serum-free medium in normoxic (20%) or low oxygen (5%) conditions for 42 days. Based on a recent finding that chondrogenesis of IFP derived MSCs in pellet culture is enhanced in hypoxic conditions¹⁵, we hypothesised that a low oxygen environment (5%) would enhance the functional properties of cartilaginous tissues engineered using IFP derived MSCs embedded in agarose hydrogels.

Materials and methods

Cell isolation and expansion

CCs were isolated from cartilage slices obtained from porcine femoral condyles (4 month old, ~50 kg, two donors) *via* digestion with collagenase type II (0.5 mg/ml) (Sigma–Aldrich, Dublin, Ireland) for 16–18 h under constant rotation at 37 °C. Cells were seeded at an initial density of 50×10^3 cells/cm² in T-175 flasks and expanded at a ratio of 1:2 to passage one (P1) in Dulbecco's Modified Eagle Medium and Ham's F12 (DMEM/F-12, Sigma Aldrich, Dublin, Ireland) supplemented with 10% v/v foetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (GIBCO, Biosciences, Dublin, Ireland).

Porcine IFP was harvested from the same donors as for CC, washed with phosphate buffered saline (PBS), diced and digested in the same manner as CCs. Mononucleated cells (MNCs) were plated in T-175 cm² flasks (Sarstedt, Wexford, Ireland) at a density of 5×10^3 cells/cm² and cultured in expansion medium (EM) consisting of high glucose Dulbecco's Modified Eagle Medium (hgDMEM) containing 10% FBS and 1% penicillin (100 U/ml)-streptomycin (100 μ g/ml) (all from GIBCO, Biosciences, Dublin, Ireland). Cultures were expanded to passage three (P3, ~21 days from initial isolation) at a seeding density of 5×10^3 cells/cm² at each passage in EM.

Hydrogel construct fabrication and culturing

Culture-expanded cells (CC and IFP MSCs) were encapsulated in agarose (Type VII) at ~40 °C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/ml to produce cylindrical discs (\emptyset 5 mm \times 3 mm thickness). Constructs were maintained in a chemically defined medium (CDM) consisting of DMEM Gluta-MAX™ supplemented with penicillin (100 U/ml)–streptomycin (100 μ g/ml) (both GIBCO, Biosciences, Ireland), 100 μ g/ml sodium pyruvate, 40 μ g/ml L-proline, 50 μ g/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1 \times insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich, Ireland) with (CC+, IFP+) and without (CC–) 10 ng/ml recombinant human TGF- β 3 (ProSpec-Tany TechnoGene Ltd, Israel) supplementation. Constructs were exposed to either normoxic (20%) or low (5%) oxygen tension for 42 days.

Assessment of functional properties

Constructs were mechanically tested in unconfined compression using a standard materials testing machine with a 5 N load cell (Zwick Z005, Roell, Germany) as previously described^{7,74}. Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 0.001 mm/s until 10% strain was obtained and maintained until equilibrium was reached. A relaxation time of 30 min was allowed. The compressive equilibrium modulus was determined using the equilibrium force. For dynamic tests, a cyclic strain of 1% was applied for 10 cycles at 0.1 Hz and 1 Hz. Dynamic moduli at each frequency were calculated through the ratio of the determined stress amplitude and the applied strain amplitude. Constructs were cored using a 3 mm biopsy punch and separated

from the annulus; the wet mass of both the core and annulus was recorded and frozen for subsequent biochemical analyses.

Quantitative biochemical analysis

Samples were digested in papain (125 µg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60 °C under constant rotation for 18 h. Total DNA content was measured using a Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Biosciences) with a lambda DNA standard. Sulfated glycosaminoglycan (sGAG) content was estimated using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulfate standard. Collagen was determined by measuring the hydroxyproline content⁷⁵ with a hydroxyproline-to-collagen ratio of 1:7.69⁷⁶.

Histology and immunohistochemistry

Constructs were fixed in 4% paraformaldehyde overnight, rinsed in PBS, and embedded in paraffin. The constructs were embedded such that sectioning at 8 µm produced a cross-section perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1 M HCl which stains for glycosaminoglycans (GAGs), and picro-sirius red to stain collagen. The deposition of collagen types I and II was identified through immunohistochemistry as previously described. Briefly sections were treated with chondroitinase ABC (Sigma) in a humidified environment to enhance permeability of the ECM by removal of chondroitin sulfate. Slides were rinsed with PBS, quenched of peroxidase activity, and blocked with goat serum for 2 h. Sections were then incubated overnight at 4 °C with mouse monoclonal collagen type I diluted 1:400 (Abcam, UK) (concentration 5.4 mg/ml) or mouse monoclonal anti-collagen type II diluted 1:100 (Abcam, UK) (concentration 1 mg/ml). After washing in PBS, the secondary antibody for type I and type II collagen (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/l) binding was used for 1 h. Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, UK) for 45 min and 5 min exposure to Peroxydase DAB substrate kit (Vector

laboratories, UK). Positive and negative controls (porcine cartilage and ligament) were included in the immunohistochemistry staining protocol for each batch (see Supplementary Fig. S1).

Statistical analysis

Statistical analysis was performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance with factors of culture time, cell source, construct region (annulus–core) and culturing regime (normoxia or low oxygen), and interactions between these factors examined. Bonferroni post-tests for multiple comparisons were used to compare conditions. Table I provides sample numbers for biochemical analysis. Three samples were assessed for mechanical properties at each time point. Numerical and graphical results are displayed as mean ± 95% confidence interval (CI). Significance was accepted at a level of $P < 0.05$.

Results

Total DNA content increased with time for CC+ constructs under normoxic (20%) conditions reaching a maximum by day 42, and was significantly higher than CC– ($P < 0.0001$) constructs at day 42 [Fig. 1(A)]. This steady increase in DNA content was not observed for CC+ constructs cultured under low oxygen (5%) conditions. DNA content of CC– constructs decreased from day 0 levels for both normoxic ($P < 0.0001$) and low ($P = 0.0015$) oxygen tensions.

Total sGAG content of CC+ constructs was higher than both CC– and IFP+ groups at day 42 under normoxic (20%) conditions ($P < 0.0001$) [Fig. 1(B)]. However, under low oxygen (5%) tension, CC+ constructs exhibited the least sGAG accumulation by day 42 compared to both CC– ($P < 0.0001$) and IFP+ ($P < 0.0001$) groups. Interestingly, under low oxygen conditions, CC– constructs exhibited the highest sGAG levels by day 42. IFP constructs attained near equivalent levels of sGAG content which were also higher than similar constructs cultured under normoxic conditions ($P < 0.0001$). When normalised by DNA content [Table I and Fig. 1(C)], no differences were found between GAG/DNA levels for CC+ and CC– constructs under normoxic conditions at day 42. In contrast, for low oxygen groups, CC+ constructs again

Table I

Total and regional (annulus/core) biochemical constituents (sGAG and collagen, normalised by DNA) at day 0, 21 and 42 for CC (CC+: with TGF-β3, CC–: without TGF-β3) and IFP (IFP+: with TGF-β3) seeded constructs subjected to 20% or 5% oxygen tension. Data is presented as the mean with 95% CIs. Sample numbers are provided in brackets. (– indicates measurement was below the detection limit of the assay). Statistical significance: (a) compared to corresponding core at the same oxygen tension (b) compared to same geometry or geometric region at other oxygen tension

| Type | Constituent | Region | Day 0 | Day 21 | | Day 42 | |
|------|--------------|---------|---------------|--|--|--|--|
| | | | | 20% | 5% | 20% | 5% |
| CC+ | sGAG/DNA | Total | 6.0 ± 0.7 (4) | 103.1 ± 12.4 (4) | 109.8 ± 42.9 (4) | 112.2 ± 11.9 (4) | 56.8 ± 7.9 (3) |
| | | Annulus | 5.5 ± 1.1 (4) | 100.3 ± 15.7 (4) | 109.0 ± 58.3 (4) | 107.2 ± 12.7 (4) | 46.5 ± 6.5 (4) ^a ($P=0.0083$), ^b ($P<0.0001$) |
| | | Core | 7.0 ± 0.5 (4) | 111.2 ± 5.4 (4) | 117.1 ± 28.5 (4) | 124.8 ± 9.1 (4) ^b ($P<0.0001$) | 82.2 ± 16.8 (3) |
| | Collagen/DNA | Total | – | 31.5 ± 3.1 (4) | 27.6 ± 8.0 (4) | 41.0 ± 6.3 (4) | 18.3 ± 11.7 (3) |
| | | Annulus | – | 31.3 ± 4.2 (4) | 29.2 ± 11.0 (4) | 43.2 ± 10.2 (4) ^b ($P=0.0421$) | 20.6 ± 8.0 (3) |
| | | Core | – | 32.2 ± 1.8 (4) | 25.5 ± 7.2 (4) | 35.6 ± 8.6 (4) ^b ($P=0.0238$) | 14.0 ± 30.5 (3) |
| CC– | sGAG/DNA | Total | 6.0 ± 0.7 (4) | 70.0 ± 4.0 (4) | 105.5 ± 11.9 (4) ^b ($P=0.0002$) | 108.9 ± 23.2 (4) | 160.8 ± 20.9 (4) |
| | | Annulus | 5.5 ± 1.1 (4) | 59.2 ± 6.3 (4) | 99.4 ± 16.1 (4) ^b ($P=0.0374$) | 90.8 ± 26.0 (4) ^a ($P<0.0001$) | 153.7 ± 25.8 (4) ^b ($P<0.0001$) |
| | | Core | 7.0 ± 0.5 (4) | 91.1 ± 13.5 (4) | 119.3 ± 21.8 (4) | 140.9 ± 18.6 (4) ^b ($P<0.0007$) | 175.9 ± 10.7 (4) |
| | Collagen/DNA | Total | – | 12.0 ± 2.5 (4) | 21.2 ± 1.4 (4) ^b ($P=0.0248$) | 20.5 ± 8.5 (4) | 33.1 ± 4.2 (4) |
| | | Annulus | – | 9.3 ± 2.1 (4) | 20.2 ± 1.9 (4) ^b ($P=0.0087$) | 16.8 ± 6.1 (4) | 33.1 ± 4.7 (4) |
| | | Core | – | 17.3 ± 5.7 (4) | 23.1 ± 0.8 (4) | 27.0 ± 13.5 (4) | 33.2 ± 3.3 (4) |
| IFP+ | sGAG/DNA | Total | 1.4 ± 0.2 (3) | 26.6 ± 3.3 (3) | 67.8 ± 6.8 (3) ^b ($P=0.0002$) | 80.6 ± 13.4 (3) | 118.1 ± 6.4 (4) |
| | | Annulus | 1.6 ± 0.4 (3) | 19.3 ± 3.9 (3) | 56.6 ± 4.0 (3) | 55.8 ± 15.6 (3) ^a ($P<0.0001$) | 107.2 ± 5.7 (4) ^a ($P=0.0018$), ^b ($P<0.0001$) |
| | | Core | 1.1 ± 0.6 (3) | 39.8 ± 3.6 (3) | 86.4 ± 18.3 (3) ^b ($P=0.0241$) | 112.9 ± 21.3 (3) ^b ($P=0.0469$) | 140.0 ± 8.3 (4) |
| | Collagen/DNA | Total | – | 35.1 ± 3.5 (3) | 42.0 ± 3.3 (3) | 74.7 ± 8.3 (3) | 82.1 ± 11.4 (4) |
| | | Annulus | – | 30.7 ± 4.0 (3) ^a ($P=0.0014$) | 39.5 ± 4.9 (3) | 51.6 ± 6.6 (3) ^a ($P<0.0001$) | 79.7 ± 16.3 (4) ^b ($P=0.0008$) |
| | | Core | – | 43.1 ± 4.4 (3) | 46.2 ± 9.0 (3) | 105.3 ± 33.7 (3) | 86.8 ± 8.2 (4) |

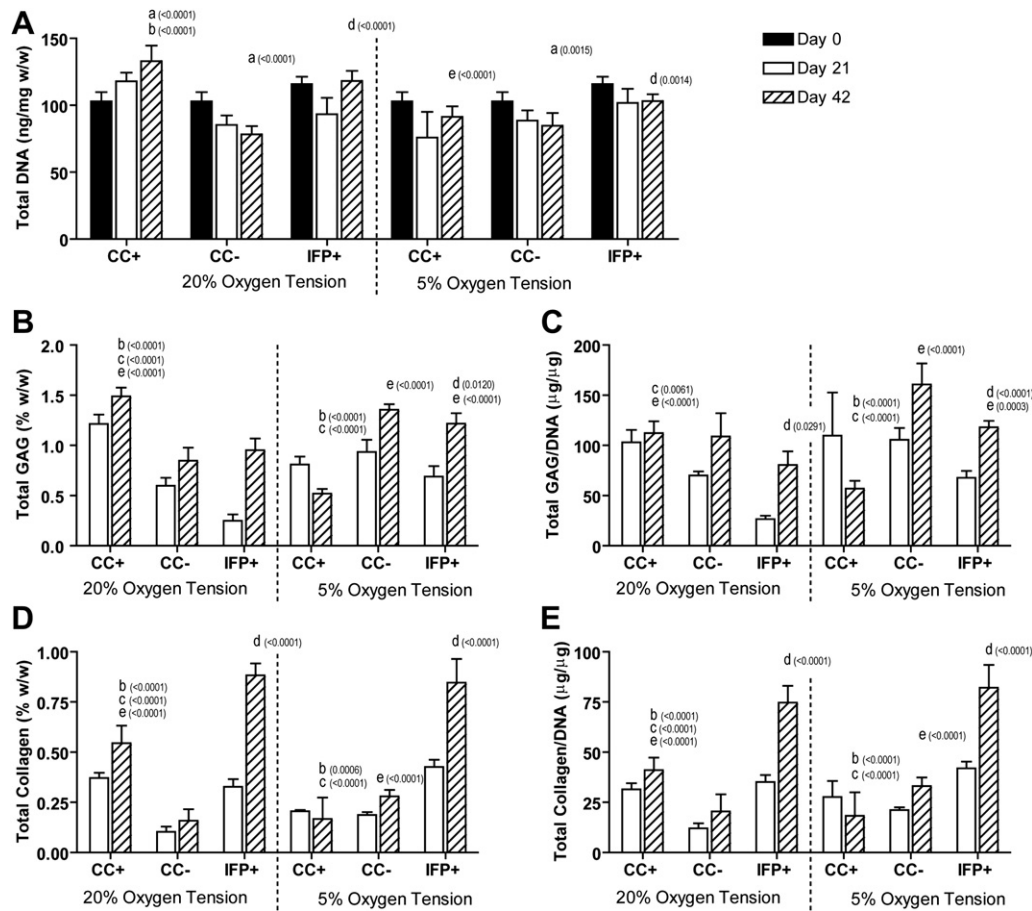


Fig. 1. Total biochemical analysis of CC (CC+: with TGF- β 3, CC-: without TGF- β 3) and IFP (IFP+: with TGF- β 3) seeded constructs subjected to 20% (LHS) or 5% (RHS) oxygen tensions at day 0, 21 and 42. (A) DNA content (ng/mg w/w) (B) GAG content (% w/w) (C) GAG/DNA content (μ g/ μ g) (D) collagen content (% w/w) and (E) collagen/DNA content (μ g/ μ g). Statistical significance: (a) compared to similar group at day 0, (b) CC+ compared to CC-, (c) CC+ compared to IFP+ (d) IFP+ compared to CC- for the same oxygen tension and time point, (e) compared to same cell group at other oxygen tension at the same time point. Data is presented as the mean with error bars representing 95% CI, $n = 3-4$ samples for each group at each time point. P values are provided in brackets.

demonstrated the lowest levels compared to CC- ($P < 0.0001$) and IFP ($P < 0.0001$) groups.

With respect to collagen content, for both normoxic and low oxygen conditions, IFP+ constructs demonstrated superior accumulation compared to both CC+ ($P < 0.0001$) and CC- groups ($P < 0.0001$) (Fig. 1D). Equivalent levels of collagen accumulation within IFP constructs were found for both normoxic and low oxygen conditions. Collagen accumulation was lowest for CC+ constructs maintained at a low oxygen tension by day 42. These trends were also reflected when normalised by DNA content [Table 1 and Fig. 1(E)].

The cores and annuli of constructs were assessed separately to gain an appreciation of the spatial distribution of biochemical constituents (Fig. 2). The supplementation of TGF- β 3 (+ groups) had an overall effect in promoting cellular proliferation for normoxic conditions. By day 42 under normoxic conditions, greater cellular proliferation had occurred in the annulus of CC+ constructs compared to respective cores ($P < 0.0001$) which were not significantly different from day 0 levels (Fig. 2A). No spatial differences were observed for CC- constructs; with lower levels at day 42 compared to day 0 indicating some cell death had occurred. For IFP+ constructs, higher cellular proliferation occurred in the core regions of constructs ($P = 0.0065$) under normoxic conditions.

By day 42, under normoxic conditions higher core levels of sGAG were attained for both CC- ($P < 0.0001$) and IFP+ ($P < 0.0001$)

groups compared to their respective annuli [Fig. 2(B,C)], with similar trends observed under low oxygen tensions. In contrast, for CC+ constructs under normoxic conditions, higher annular sGAG accumulation was observed compared to the corresponding core ($P = 0.0038$) [Fig. 2(B)].

For collagen content, IFP+ constructs demonstrated significantly higher core levels (~ 2.4 fold) compared to respective annuli under normoxic conditions but no differences were observed in the spatial distribution for low oxygen tension constructs with lower core ($P < 0.001$) and higher annular ($P = 0.0106$) levels when directly compared to regions under normoxic conditions [Fig. 2(D)]. This development of a more homogeneous collagen distribution for IFP+ constructs under low oxygen tension was also seen when normalised by DNA content [Table 1 and Fig. 2(E)]. Overall for both culture conditions, IFP+ constructs attained the highest core levels of collagen compared to all other groups.

Histological evaluation revealed that constructs stained positively for sulphated proteoglycan and collagen accumulation for all groups under both culture conditions (Fig. 3). Intense staining of sGAGs and collagen type II was observed for both CC+ and IFP+ constructs, with 5% IFP+ constructs exhibiting more intensive staining for collagen type II compared to all groups (Fig. 3, bottom row). Minimal collagen type I staining was observed for CC- and IFP+ groups at 20% oxygen tension, while there did not appear to be any significant staining for any of the groups subjected to low oxygen tension (Fig. 3).

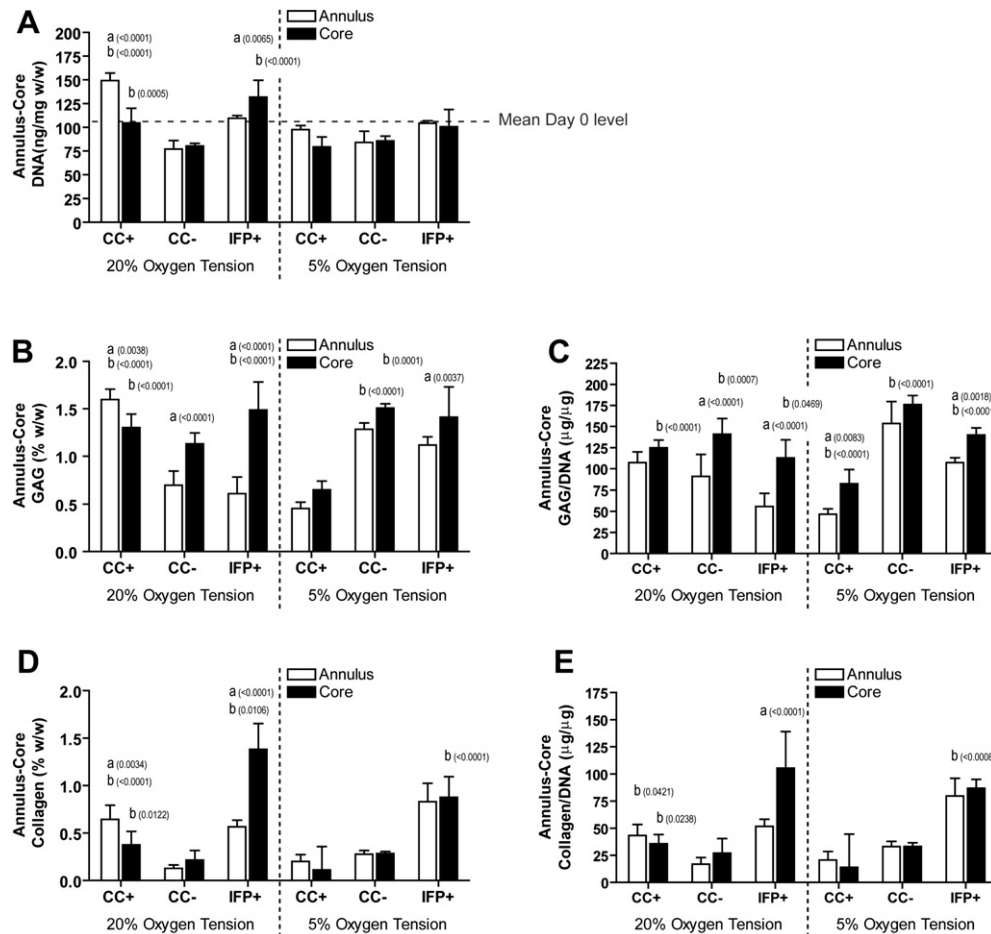


Fig. 2. Annulus–core biochemical analysis of CC (CC+: with TGF- β 3, CC–: without TGF- β 3) and IFP (IFP+: with TGF- β 3) seeded constructs subjected to 20% (LHS) or 5% (RHS) oxygen tensions at day 42. (A) DNA content (ng/mg w/w) (B) GAG content (% w/w) (C) GAG/DNA content (μ g/ μ g) (D) collagen content (% w/w) and (E) collagen/DNA content (μ g/ μ g). Statistical significance: (a) compared to corresponding core at the same oxygen tension (b) compared to same geometric region at other oxygen tension at day 42. Data is presented as the mean with error bars representing 95% CI, $n = 3$ –4 samples for each group. P values are provided in brackets.

Under normoxic (20%) conditions CC+ constructs demonstrated superior mechanical properties compared to both IFP+ and CC– groups ($P < 0.0001$) for both equilibrium and dynamic moduli by day 42 (Fig. 4). No differences in equilibrium modulus were found comparing IFP+ and CC– groups, but differences did exist for dynamic moduli ($P < 0.0001$).

In contrast, under low oxygen tension (5%) CC+ constructs demonstrated inferior mechanical properties compared to both CC– ($P < 0.0001$) and IFP+ ($P < 0.0001$) groups by day 42, which were also significantly inferior to CC+ constructs cultured under normoxic conditions (~ 3.5 fold, $P < 0.0001$). Furthermore, both CC– and IFP+ groups had superior mechanical properties when cultured under low oxygen tensions compared to normoxic conditions ($P < 0.0001$). The dynamic modulus of IFP+ constructs was higher than both CC+ and CC– groups in low oxygen conditions ($P < 0.0001$).

Discussion

Articular cartilage is an avascular tissue with the primary mode of nutrient delivery (e.g., oxygen, glucose) supplied to CCs via diffusion from the synovial fluid. It has been estimated that *in vivo* oxygen tensions decrease with depth from the cartilage surface, with the gradient dependent on the rate of oxygen transport through cartilage and the rate of cellular consumption⁷⁷. Mathematical models have predicted that the oxygen tension at the

cartilage superficial zone decreases with depth from 5% at the superficial zone to approximately 1% in the subchondral bone region⁷⁷ while experimental models have estimated that the oxygen tension varies between 7% and 1%⁷⁸. The objective of this study was to compare the functional properties of cartilaginous tissues engineered in either a low oxygen (5%) or normoxic (20%) environment using CCs and IFP derived MSCs. The results of this study corroborate the hypothesis that the biomechanical functionality of IFP derived tissues generated in a low oxygen environment is superior to that generated in a normoxic environment. Hypoxic conditions have previously been shown to enhance chondrogenesis of IFP derived MSCs in pellet culture¹⁵. The results of this study demonstrate that such findings from pellet studies can be translated to tissue engineering models incorporating scaffolds or hydrogels. In the context of cell-based therapies for cartilage repair, two important conclusions can be drawn from the results of this study. In the context of *ex vivo* cartilage tissue engineering, a more functional cartilaginous tissue can be engineered using CCs supplemented with TGF- β 3 in a normoxic environment. However, when viewed as a model of how both cell types may respond to a low oxygen environment, the results suggest that a repair tissue generated by IFP derived MSCs could be superior to that generated using CCs.

Matrix production by CCs embedded in agarose hydrogels is enhanced in a reduced oxygen environment when cultured in the absence of TGF- β 3. The finding that low oxygen conditions are

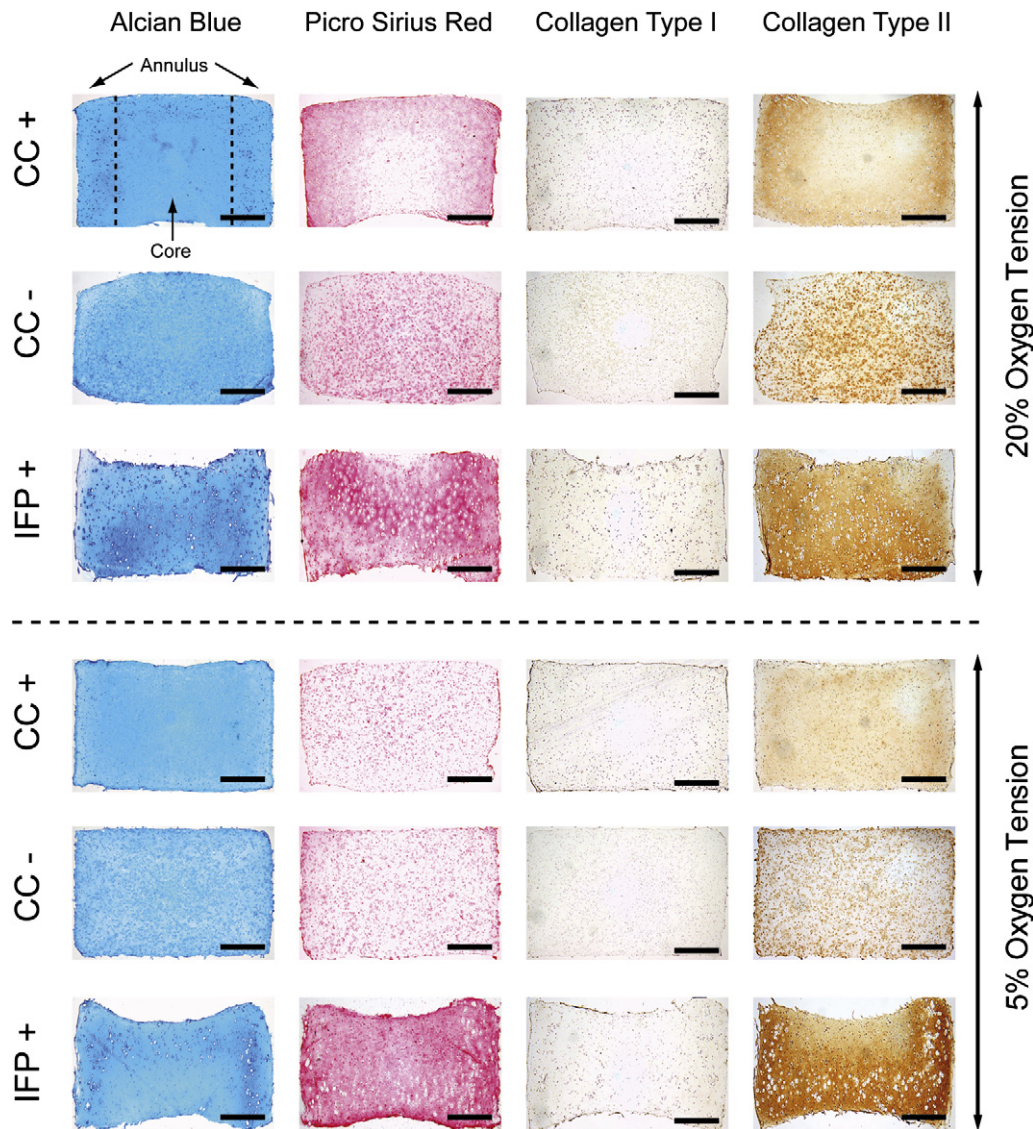


Fig. 3. Histological evaluation of CC (CC+: with TGF- β 3, CC-: without TGF- β 3) and IFP (IFP+: with TGF- β 3) seeded constructs subjected to 20% (Top) or 5% (Bottom) oxygen tension at day 42. Alcian blue staining for sulphated proteoglycan, picro-sirius red for total collagen and immunohistochemistry for collagen types I and II. Images are representative of full central cross-sections of constructs; annulus–core region is indicated in top left image. Scale bar is 1 mm.

beneficial is in agreement with earlier studies that demonstrated a reduced oxygen tension supports the chondrogenic phenotype^{56–58,63,65}. A reduction in DNA content was also observed at both oxygen tension levels in the absence of TGF- β 3, and at 5% oxygen in the presence of TGF- β 3. For CCs cultured in the absence of TGF- β 3, this reduction in CC proliferation at a 5% oxygen tension is accompanied by a dramatic increase in GAG/DNA levels, suggesting a change in cellular metabolism that has also been observed for adipose tissue derived MSCs in a low oxygen environment⁷¹.

Interestingly, a low oxygen environment was observed to inhibit cartilage matrix production by CCs compared to normoxic conditions when cultured in the presence of TGF- β 3. The literature provides evidence for hypoxia both promoting and inhibiting chondrogenesis⁵⁵. For example, it has been demonstrated that proteoglycan production by growth plate CCs is greater at aerobic conditions⁶², and similarly elevated proteoglycan synthesis in mature bovine articular explant cultures was observed at 24% oxygen compared to 5%⁵². In the context of cartilage tissue engineering, it has also been suggested that more aerobic conditions are

beneficial for robust ECM production^{60,61}. The results of the present study (which demonstrate a differential response to hypoxia by CCs cultured in the presence or absence of TGF- β 3) suggest that the response of CCs to hypoxia may be modulated by TGF- β . CCs isolated from the growth plate, an avascular tissue, have been shown to secrete TGF- β as a means to inhibit neo-angiogenesis⁷⁹. Therefore the supplementation of additional TGF- β to the media may be interfering with the normal response of CCs to a low oxygen environment *via* a negative feedback process. Further experimentation is required to test this hypothesis.

Continuous supplementation of TGF- β 3 is also known to have a negative effect on the development of a functional cartilaginous tissue engineered using bovine CCs, compared to conditions whereby the growth factor is withdrawn from the culture media after 2 weeks³⁹. The authors of that study suggested that TGF- β 3 may act to prime the cells and thereby enable the robust and rapid accumulation of matrix molecules after its effects have dissipated³⁹. The superior response of CCs to a low oxygen environment in the absence of TGF- β 3 could possibly be explained in a similar

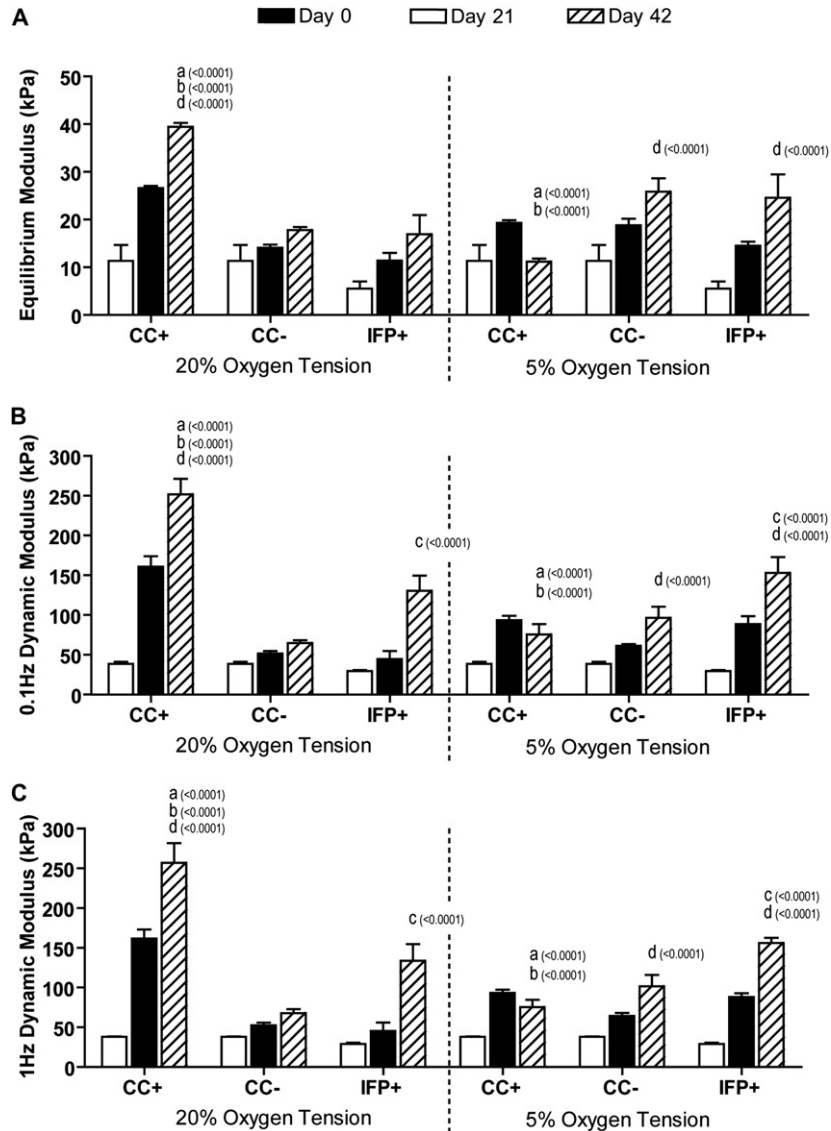


Fig. 4. Mechanical properties of CC (CC+: with TGF- β 3, CC-: without TGF- β 3) and IFP (IFP+: with TGF- β 3) seeded constructs subjected to 20% (LHS) or 5% (RHS) oxygen tensions at day 0, 21 and 42. (A) Equilibrium modulus (B) 0.1 Hz dynamic modulus and (C) 1 Hz dynamic modulus. Statistical significance (a) CC+ compared to CC-, (b) CC+ compared to IFP+ (c) IFP+ compared to CC- for the same oxygen tension and time point, (d) compared to same cell group at other oxygen tension at day 42. Data is presented as the mean with error bars representing 95% CI, $n = 3$ samples for each group at each time point. P values are provided in brackets.

manner, where the continued presence of TGF- β 3 is in some way inhibiting the rapid accumulation of sGAG in response to the chondrogenic stimulus provided by the low oxygen environment. This hypothesis could be tested by subjecting cell seeded constructs to temporal TGF- β 3 supplementation in normoxic and low oxygen environments.

We have previously suggested that the greater sGAG accumulation in the core of cartilaginous tissues engineered using either bone marrow or IFP derived MSCs could be due to a lower oxygen tension within this region of the construct⁷. Numerical models have previously been used to demonstrate that gradients in oxygen tension exist within tissue engineered constructs due to cellular consumption and/or utilization of the molecule^{77,80,81}. Interestingly the difference between core and annulus matrix production partially diminishes in a lower oxygen tension environment. The development of an inhomogenous cartilaginous construct can also reduce the apparent mechanical properties of the construct determined from compression tests⁸². It is also noted that sGAG accumulation is still higher in the core of IFP constructs, suggesting

that oxygen tension levels below 5% may in fact be more chondrogenic. Further studies at lower oxygen tension levels are required to optimise this key regulatory factor for cartilage tissue engineering.

While the mechanical properties of the engineered cartilaginous grafts are still an order of magnitude lower than that of native cartilage tissue, the results of this study demonstrate how environmental factors such as oxygen tension differentially regulate the functional properties of tissue derived from CCs and MSCs. Future studies will explore if alteration of experimental conditions such as the initial cell density or growth factor supplementation can improve the subsequent functional properties of engineered grafts. In addition, this study utilised cells from two donors. There are small differences in MSC proliferation between this study and previous studies from our lab¹⁶, which may be related to donor variability. Future studies are therefore required to explore donor variability in response to low oxygen conditions. Other areas for future investigation include using real-time polymerase chain reaction and appropriate biochemical assays to quantify the

relative amounts of different types of collagen (e.g., type I vs II) formed in response to altered oxygen conditions.

In conclusion, the results of this study suggest that oxygen tension regulates chondrogenesis of both IFP derived MSCs and CCs. The beneficial response of CCs to a low oxygen environment in the absence of TGF- β suggests that hypoxia can be used as an alternative to growth factor stimulation to engineer cartilage from culture-expanded CCs. Perhaps of greater interest is the differential response of CCs and IFP derived MSCs to a low oxygen environment in the presence of TGF- β . Previous studies have consistently demonstrated that MSCs are inferior to CCs as a cell source for functional cartilage tissue engineering^{6,83}. To the best of our knowledge this is the first study to demonstrate that IFP derived MSCs produce a more mechanically functional tissue than CCs in a low oxygen environment. Cells or engineered tissues that are delivered to damaged or diseased joints for therapeutic purposes are subjected to a low oxygen tension and a milieu of growth factors including members of the TGF- β superfamily^{84–87}. Extrapolating the results of this study to the *in vivo* setting, it would appear that joint fat pad derived MSCs may possess a superior potential to generate a functional repair tissue in such an environment.

Author contributions

CTB and DJK designed experiment and prepared manuscript. CTB and TV performed all experimental work and data analysis.

Conflict of interest

The authors certify that there is no conflict of interest related to the work presented in this manuscript.

Acknowledgements

Funding was provided by Science Foundation Ireland (President of Ireland Young Researcher Award, 08/YI5/B1336).

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2010.07.004.

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