Extracellular Matrix Production by Nucleus Pulposus and Bone Marrow Stem Cells in Response to Altered Oxygen and Glucose Microenvironments

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Running Header: Matrix Production by Nucleus Pulposus and Bone Marrow Stem Cells

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Abstract

Bone marrow (BM) stem cells may be an ideal source of cells for intervertebral disc (IVD) regeneration. However the harsh biochemical microenvironment of the IVD may significantly influence the biological and metabolic vitality of injected stem cells and impair their repair potential. This study investigated the viability and production of key matrix proteins by nucleus pulposus (NP) and BM stem cells cultured in the typical biochemical microenvironment of the IVD consisting of altered oxygen and glucose concentrations. Culture expanded NP cells and BM stem cells were encapsulated in 1.5% alginate and ionically crosslinked to form cylindrical hydrogel constructs. Hydrogel constructs were maintained under different glucose concentrations (1mM, 5mM and 25mM) and external oxygen concentrations (5% and 20%). Cell viability was measured using the Live/Dead® assay and the production of sulphated glycosaminoglycans (sGAG) and collagen was quantified biochemically and histologically. For BM stem cells, IVD-like micro-environmental conditions (5mM glucose and 5% oxygen) increased the accumulation of sGAG and collagen. In contrast, low glucose conditions (1mM glucose) combined with 5% external oxygen concentration promoted cell death inhibiting proliferation and the accumulation of sGAG and collagen. NP-encapsulated alginate constructs were relatively insensitive to oxygen concentration or glucose condition in that they accumulated similar amounts of sGAG under all conditions. Under IVD-like microenvironmental conditions, NP cells were found to have a lower glucose consumption rate compared to BM cells and may in fact be more suitable to adapt and sustain the harsh microenvironmental conditions. Considering the highly specialised microenvironment of the central NP, these results indicate that IVD-like concentrations of low glucose and low oxygen are critical and influential for the survival and biological behaviour of stem cells. Such findings may promote and accelerate the translational research of stem cells for the treatment of IVD degeneration.

Keywords: intervertebral disc; nucleus pulposus; bone marrow; stem cells; oxygen; glucose; microenvironment; metabolism

Introduction

Low back pain (LBP) is a significant epidemiological problem and economic burden worldwide (Hoy et al., 2010). It is established that the primary cause of LBP is degeneration of the intervertebral disc (IVD) characterised by decreased extracellular matrix (ECM) synthesis and increased cell death (Deyo and Weinstein, 2001). IVD degeneration initiates within the nucleus pulposus (NP) and progresses with attrition of the annulus fibrosus (AF) which leads to eventual impairment of the IVD.

Healthy NP tissue contains randomly organized collagen types II and VI, embedded in a highly hydrated gel-like matrix rich in proteoglycans (PGs), with aggrecan being predominantly abundant (Inoue, 1981). Other proteoglycans such as biglycan, decorin, and fibromodulin are also present (Singh et al., 2009). The high osmotic pressure within the NP, provided by the proteoglycans, is important in maintaining tissue hydration and resisting compressive forces during normal motion and activities. As degeneration progresses, the proteoglycan content of the NP diminishes resulting in decreased osmotic pressure with a concomitant loss of hydration and reduction in disc height thereby impairing the mechanical functionality of the IVD. Synthesis and composition of collagens also vary with progressive degeneration; with increased collagen type I produced in the NP, leading to a fibrotic transformation of the NP tissue and a progressive inability to identify a clear demarcation between the NP and AF tissues. Concomitant with matrix degradation and reduced disc height is often an in-growth of blood vessels and nerves into the normally avascular and aneural tissue (Freemont et al., 1997).

Cell based therapies targeted to regenerate the NP region may prevent progressive degeneration. Autologous Disc Cell Transplantation (ADCT) is a therapy that involves harvesting NP tissue from the patient, isolating and expanding cells to required numbers and injecting the expanded cells into the central NP region of an early-stage degenerated IVD (Hohaus et al., 2008). However, limitations with this approach include the low yield of healthy NP cells obtainable from degenerated discs and the limited expansion capability of NP cells (Hiyama et al., 2008, Xia et al., 2013). This has motivated the exploration of stem cells due to their propensity to proliferate and their ability to form multiple tissue types (Caplan, 1991).

BM stem cells possess significant potential and perhaps provide a clinically feasible source of cells to promote the repair of NP tissue. The rationale and benefits to transplanting stem cells into the IVD are twofold; firstly, transplanted stem cells may stimulate endogenous NP cells, and secondly the resident host NP cells may promote differentiation of the transplanted stem cells towards a nucleus pulposus phenotype (Miyamoto et al., 2010, Richardson et al., 2006). In vivo studies have shown that implantation of stem cells into experimentally induced degenerate animal discs leads to improved disc height and accumulation of proteoglycans (Risbud et al., 2004a, Crevensten et al., 2004, Sakai et al., 2003). Furthermore, a human clinical study performed by Orozco et al injected autologous bone marrow stem cells into the nucleus pulposus of ten patients diagnosed with lumbar disc degeneration. Results indicated that pain, disability, and quality of life improved over the 12 month trial (Orozco et al., 2011).

However, the regenerative potential of BM stem cells may be limited by the harsh microenvironment within the disc, characterised by low oxygen, low glucose and low pH conditions (Urban, 2002, Grunhagen et al., 2006, Bartels et al., 1998). In the central nucleus pulposus the oxygen concentration ranges from 5% to as low as 1% (Mwale et al., 2011), the

pH ranges from 7.1 to as low 6.5 (Urban, 2002) and the glucose concentration ranges from 5mM to lower levels (Bibby et al., 2005) as the degeneration transgresses from mildly degenerated to a severely degenerated state. NP cells have been shown to be well adapted to this harsh microenvironment (Risbud et al., 2006), but this biochemical microenvironment may negatively influence the biological and metabolic vitality of stem cells and impair their regeneration potential. Therefore, understanding how stem cells respond to limited nutrient availability is a key factor for clinical translation.

Numerous studies have focused on cell growth and survival (Johnson et al., 2008, Stephan et al., 2011). Stephan *et al* cultured bovine NP cells in alginate beads under zero glucose or high glucose conditions and demonstrated that NP cell proliferation and survival are influenced by the availability of glucose (Stephan et al., 2011). The absence of glucose resulted in more apoptotic and senescent cells. Interestingly, Johnson *et al* cultured bovine NP cells encapsulated in alginate gels under similar conditions and observed that glucose deprivation leads to a minimal increase in cell proliferation (Johnson et al., 2008). Mwale *et al* also cultured bovine NP cells encapsulated in alginate beads under different oxygen concentrations and found that low oxygen levels increased the expression of aggrecan mRNA levels but interestingly this was not reflected in GAG release (Mwale et al., 2011). Also, Stoyanov et al cultured BM stem cells in alginate beads under low and high oxygen concentrations and observed that hypoxia increased aggrecan and collagen gene expression (Stoyanov et al., 2011). Although these studies describe the influence of glucose and oxygen on NP cell and BM stem cell growth and survival, little is known of the effect on the capacity of these cells to produce NP-like matrix. Further experimentation is required to address ECM synthesis which is of major importance to the functioning of the disc. Furthermore, the same studies have investigated the effects of oxygen (Yang et al., 2013, Stoyanov et al., 2011, Risbud et al., 2006, Mwale et al., 2011) or glucose (Wuertz et al., 2008, Stephan et al., 2011,

Liang et al., 2012, Deorosan and Nauman, 2011, Li et al., 2007) independently which has resulted in several contradictions in the literature and confirms the need to study the effect of combined environmental factors which more likely reflects the situation as it exists *in vivo.*

The objective of this study was to investigate how microenvironmental conditions may affect subsequent matrix production of porcine NP and BM stem cells encapsulated in 3D alginate hydrogels cultured in three different glucose (1mM, 5mM and 25mM) media at two different oxygen concentrations (5% and 20%).

Methods

Nucleus Pulposus and Bone Marrow Stem Cell Isolation and Culture

NP cells were harvested from the intervertebral discs (IVDs) of porcine donors (N=2, 3-4 months, 20-30kg) within three hours of sacrifice as previously described (Naqvi and Buckley, 2014). NP tissue was isolated and enzymatically digested in 2.5mg/ml pronase solution for 1 hour followed by 3 hours in 0.5mg/ml collagenase solution at 37°C. Digested tissue/cell suspension was passed through a 100µm cell strainer to remove tissue debris followed by 70 µm and 40 µm cell strainers to separate notochordal cells (NC) from the desired nucleus pulposus cells (NP) as previously described (Spillekom et al., 2014). Cells were washed three times by repeated centrifugation (650G for 5 minutes), plated at a density of 5×10^3 cells/cm^2 and cultured to passage 2 in T-175cm^2 flasks with low-glucose Dulbecco's modified eagles medium (LG-DMEM, 1mg/ml D-Glucose), supplemented with 10% Foetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin B, 5ng/ml Fibroblast Growth Factor-2 (FGF-2; PeproTech, UK).

Donor matched bone marrow (BM) was isolated from the femora and plated at 10×10^6 cells in T-75cm^2 flasks to allow for colony formation (P0) in supplemented LG-DMEM. After P0, cells were re-plated at 5×10^3 cells/cm^2 and expanded to P2 in a humidified atmosphere at 37°C and 5% CO_2. The differentiation capacity of BM cells from donors was assessed as previously described (Vinardell et al., 2011). In all cases BM stem cells demonstrated successful differentiation towards the osteogenic, adipogenic and chondrogenic lineages.

Alginate Hydrogel Encapsulation and Culture

Expanded cells (NP and BM) were encapsulated in 1.5% alginate (Pronova UP LVG, FMC NovaMatrix, Norway) at a seeding density of $4x10^6$ cells/ml and ionically crosslinked with 100mM calciumchloride ($CaCl_2$) for 30 minutes to form cylindrical constructs (diameter=5mm; height=3mm). The geometric construct dimensions used in this study were based on previous work from our laboratory (Buckley et al., 2012). Constructs were maintained in 1mM, 5mM or 25mM-glucose medium consisting of DMEM supplemented with penicillin (100 U/mL)-streptomycin (100µg/mL), (both from GIBCO, Invitrogen, Ireland), 0.25 µg/mL amphotericin B, 100 µg/ml sodium pyruvate, 40 µg/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 µg/mL linoleic acid, 1× insulin–transferrin–selenium, 50 µg/mL L- ascorbic acid-2-phosphate, 100 nM dexamethasone (all Sigma-Aldrich, Ireland) and 10 ng/mL TGF- β3 (PeproTech, UK). Constructs were cultured in standard 24 well plates with one construct per well with 2mL of supplemented medium in hypoxic (5% oxygen) or normoxic (20% oxygen) conditions. Constructs were assessed at days 0 and 21 in terms of cell viability (n=1), biochemical content (DNA, sulfated-glycosaminoglycan (sGAG) and collagen content) (n=3), histologically and immunohistochemically (n=2). This study was performed twice with independent donors in each case. Results were reproducible for all conditions investigated.

Cell Viability

Cell viability was assessed using a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen, Bio-science, Ireland). Constructs were removed from culture, sectioned, rinsed with phosphate buffered saline (PBS) and incubated for 1 hour at 37ºC in live/dead solution containing 2 µM calcein AM, 4 µM ethidium homodimer-1 (EthD-1). After incubation segments were again washed with PBS and imaged with an Olympus FV-1000 Point-

Scanning Confocal Microscope (Southend-on-Sea, UK) at 515 and 615 nm channels and analysed using FV10-ASW 2.0 Viewer software.

Quantitative Biochemical Analysis

Samples were digested with papain (125µg/ml) in 0.1M sodium acetate, 5mM L-cysteine HCl, and 0.05 M EDTA (Sigma–Aldrich, Ireland) at 60°C under constant agitation for 18 hours. DNA content was quantified using the Hoescht 33258 dye-based DNA QF Kit (Sigma-Aldrich, Ireland). Proteoglycan content was quantified using the dimethylmethylene blue (DMMB) dye-binding assay (Blyscan, Biocolor Ltd, Northern Ireland) with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Briefly, samples were hydrolysed at 110°C for 18 hours in concentrated hydrochloric acid (HCl) (38%) and assayed using a chloramine-T assay (Kafienah and Sims, 2004), using a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al., 2007).

Glucose concentrations in media samples from Day 18 to Day 21 were quantitatively measured using a glucose meter (Accu-Chek Aviva glucose meter, Roche Diagnostics Ltd, UK). Samples of culture media (1mM, 5mM and 25mM) served as controls. Cellular consumption rates were determined by normalising to cell number and time.

Histology and Immunohistochemistry

Constructs were fixed in 4% paraformaldehyde (PFA) overnight, dehydrated in ethanol, embedded in paraffin wax and sectioned at a thickness of 8 µm. Sections were stained for glycosaminoglycans (GAGs) using aldehyde fuschin and 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1M HCl (Simmons et al., 2004) and picro-sirius red to assess for

collagen deposition. The deposition of collagen types I and II were identified through immunohistochemistry. Briefly, sections were rinsed with PBS before treatment with chondroitinase ABC in a humidified environment at 37°C. Slides were rinsed with PBS and non-specific sites were blocked with goat serum. Sections were incubated for 1 hour at 4°C with the primary antibody; mouse monoclonal collagen type I antibody (1:200; 1 mg/ml) or mouse monoclonal anti-collagen type II (1:80; 1 mg/ml). After washing in PBS, sections were quenched of peroxidase activity and incubated for 1 hour in the secondary antibody; anti-mouse IgG biotin antibody produced in goats (1:133; 2.1 mg/ml). Colour was developed using the Vectastain ABC reagent followed by exposure to peroxidase DAB substrate kit. Positive and negative controls of porcine ligament and cartilage were included for each batch.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (version 4) software. Two-way ANOVA was used for analysis of variance with Bonferroni post-tests to compare between groups. Numerical and graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of $p < 0.05$.

Results

Viability of Nucleus Pulposus and Bone marrow Stem Cells in IVD-like Microenvironmental Conditions

For BM constructs, DNA content increased from day 0 for both oxygen concentrations irrespective of glucose condition (Fig 1A). A similar result was obtained for NP constructs maintained in 5mM glucose under normoxic conditions (20% oxygen). These results were confirmed through confocal imaging of live and dead cells (Figure 1B, C). Interestingly, a core of dead cells was observed in all BM constructs irrespective of the culture condition, although this core effect was more pronounced in the group maintained in 1mM glucose under hypoxia. A similar core effect was observed in NP constructs maintained in 5mM glucose in normoxia. The increase in DNA content in BM constructs from day 0 to day 21 may be due to increased cell proliferation in the periphery of constructs.

Bone Marrow Stem Cells Accumulated Greater Amounts of sGAG and Collagen in NP-like Microenvironmental Conditions

A differential response for total sGAG content was observed for NP and BM constructs depending on oxygen concentration (Figure 2A). Under normoxic conditions, total sGAG content was significantly higher for NP compared to BM constructs. In contrast, hypoxia promoted the highest total sGAG accumulation for BM constructs maintained in IVD-like microenvironmental conditions (5mM glucose and 5% oxygen). When normalised to DNA content, NP constructs displayed similar amounts of sGAG content under all conditions which correlated highly with aldehyde fuschin staining of the constructs where NP constructs appeared to accumulate similar amounts of sGAG irrespective of glucose condition or oxygen concentration (Figure 2B). Of note, BM constructs maintained in 1mM glucose in hypoxia accumulated sGAG in the periphery of the gel confirming the cell

viability results where dead cells were located in the centre of the hydrogel construct. Also, BM constructs maintained in normoxia exhibited sGAG deposition in the pericellular region only.

In terms of collagen accumulation NP constructs maintained in very low glucose conditions in hypoxia exhibited significantly less collagen content compared to those maintained in normoxia (Figure 3A). BM constructs maintained in 1mM glucose in normoxia demonstrated significantly higher collagen content compared with those maintained in hypoxia and similar results were obtained when collagen content was normalised to DNA. These observations correlated highly with picro-sirius red staining of constructs (Figure 3B). NP constructs accumulated limited but similar amounts of collagen irrespective of oxygen or glucose condition. Of note, BM constructs maintained in 1mM glucose and in hypoxia displayed limited collagen accumulation. Also, collagen staining of BM constructs maintained in normoxia demonstrated pericellular collagen deposition only.

In terms of sGAG to collagen ratio, hypoxia resulted in higher ratios for both cell types, with the highest ratios observed for NP (Figure 3C). Also, for BM constructs maintained in hypoxia, there was a~~n observed~~ decrease in sGAG:Collagen ratio with increasing glucose concentration.

Immunohistochemistry results revealed that BM constructs in 25mM glucose in hypoxia resulted in increased accumulation of collagen type I compared to normoxia (Figure 4A). In contrast, for NP constructs under the same glucose conditions; those maintained in normoxia resulted in increased accumulation of collagen type I compared to hypoxia.

In terms of collagen type II deposition, BM constructs deposited greater amounts when maintained in IVD-like microenvironmental conditions (5mM glucose and 5% oxygen) with limited deposition under ischemic conditions (very low glucose and hypoxia) (Figure

4B). Also, NP constructs maintained in ischemic conditions (very low glucose and hypoxia) exhibited significantly less deposition of collagen type II compared to normoxia (Figure 4B).

Glucose Consumption Rate of Bone Marrow Stem Cells in IVD-like Microenvironmental Conditions

BM constructs maintained in IVD-like microenvironmental conditions (5mM glucose and 5% oxygen) displayed a significantly higher glucose consumption rate ($p<0.01$) compared to NP constructs maintained under the same conditions. In addition, both cell types exhibted a significantly higher glucose consumption rate ($p<0.01$) compared to constructs maintained in normoxic conditions (Figure 5). Interestingly, under hypoxic conditions, BM constructs maintained in very low glucose (1mM) exhibited a lower consumption rate compared to BM constructs under 5mM and 25mM glucose conditions ($p<0.01$). Furthermore, under hypoxic conditions, BM constructs maintained in high glucose (25mM) exhibited a significantly higher glucose consumption rate compared to those maintained in normoxia and NP constructs irrespective of external oxygen concentration.

Discussion

The IVD is an avascular organ relying on diffusion of essential nutrients such as oxygen and glucose through the endplate thereby creating a challenging biochemical microenvironment. Translation of stem cell therapies into a multimodal protocol for IVD degeneration requires not only the survival of these cells but also their ability to function normally amidst the harsh microenvironment of hypoxia, low nutrition, acidic pH, high mechanical loading, high osmolarity, and a complicated protease and cytokine network (Wuertz et al., 2008, Urban, 2002). In this study we investigated the influence of external oxygen concentration (5% and 20% O_2) and three different glucose concentrations (1mM, 5mM and 20mM) on bone marrow (BM) stem cells and nucleus pulposus (NP) cells encapsulated in 3D alginate hydrogels.

We found that BM stem cells survive and synthesize appropriate matrix components such as sGAG and collagen in low external oxygen of 5% and low glucose concentration of 5mM representative of IVD microenvironmental conditions. Under the same external oxygen concentration of 5% and a very low glucose concentration (1mM), BM viability was reduced, particularly in the core region where we also observed reduced accumulation of matrix components (sGAG and collagen). Importantly, the GAG:Collagen ratio was relatively higher in BM constructs maintained in hypoxia compared to normoxia. A high GAG:collagen ratio may provide an appropriate metric of identifying an NP-like tissue type. The healthy NP contains randomly organized collagen type II (Inoue, 1981), embedded in a highly hydrated gel-like matrix rich in proteoglycans (PGs), with aggrecan being predominantly abundant resulting in a high GAG:collagen ratio. Conversely, the degenerated NP loses its proteoglycan content with synthesis and composition of collagens also varying resulting in an altered GAG:collagen ratio. If stem cells are to be differentiated towards a disc cell phenotype, it will be essential to verify that the ultimate matrix that they produce has an

appropriate GAG to collagen ratio which, for native NP, is approximately 3.5:1 (Mwale et al., 2004). While this ratio may not help in determining whether ultimate differentiation has occurred, it provides an indication for the correct composition of the tissue that the cells produce (Mwale et al., 2004).

Glucose is a source of energy that markedly affects viability, proliferation and differentiation of stem cells. It should be noted that cells were encapsulated in a 3D alginate hydrogel (3mmHx5mmØ) thus, for those gels maintained in hypoxia this 3D geometry has an effect of further reducing the oxygen concentration in the core due to cellular consumption (Buckley et al., 2012). This may explain the observed cell death due to inadequate oxygen combined with diminished glucose availability limiting homogeneous deposition of extracellular matrix which has been previously reported for bone marrow stem cells undergoing chondrogenesis (Farrell et al., 2012).

The results from this study illustrate that BM constructs exhibit an increased glucose consumption rate under hypoxic conditions compared to normoxic. Interestingly, the same constructs exhibit an increased glucose consumption rate compared to NP constructs under the same external oxygen tension demonstrating that NP cells consume less glucose than BM cells which suggests that NP cells can more readily adapt to changes in microenvironmental conditions. These findings provide important insight in the development of clinical cell-based therapies in determining the suitability of specific cell types for targeted regeneration.

Indeed, it has previously been reported that glucose uptake is increased when maintained in hypoxia and that stem cells are known to possess the ability to adapt their oxygen consumption rate to changes in the oxygen environment (Pattappa et al., 2013). Deschepper *et al* previously demonstrated that stem cells can remain viable when maintained in severe hypoxic conditions (i.e. 0.2% O_2) but not in the absence of glucose (Deschepper et al., 2010). This correlates well with the results from this study where BM-encapsulated gels

maintained in hypoxia with sufficient glucose (5mM and 25mM) demonstrated higher cell viability, sGAG and collagen accumulation. Importantly, the reduced cell viability was not evident in BM-encapsulated alginate hydrogels maintained under the same very low glucose concentration (1mM glucose) and an external oxygen concentration of 20%. Under these conditions, cells appeared to remain viable but only deposited matrix pericellularly. Furthermore, this was observed for all BM-encapsulated alginate hydrogels maintained under 20% oxygen conditions. Interestingly, the therapeutic potential of stem cells is commonly investigated under 20% O_2 (normoxia) conditions in vitro while the typical physiological oxygen concentration in human ranges from 4% to 7% (Packer and Fuehr, 1977, Kofoed et al., 1985) and falls to 1% in some pathological ischemic tissues, as well as in the degenerated IVD (Bartels et al., 1998). Numerous studies have investigated the influence of hypoxia and have found that BM stem cells proliferated more rapidly, exhibited greater colony forming unit (CFU) formation ability (Grayson et al., 2006, Grayson et al., 2007), and maintained better "stemness" in hypoxia through the down regulation of E2A-p21 by HIF-1a-Twist pathway (Tsai et al., 2011). Furthermore, previous studies have demonstrated that glucose is a significant factor in the metabolic response of mesenchymal stem cells (Deorosan and Nauman, 2011) and that a low oxygen environment enhances GAG synthesis in pellets and hydrogels (Sheehy et al., 2012). Risbud *et al*. found that 2% O_2 and 10 ng/ml TGF-β could stimulate rat BM stem cell differentiation to acquire phenotypes similar to that of NP cells (Risbud et al., 2004b).

In contrast, NP-encapsulated alginate hydrogels maintained under the same very low glucose concentration (1mM glucose) and external oxygen concentration of 5% did not exhibit reduced cell viability. In fact, NP cells remained relatively insensitive to external microenvironmental conditions such that similar amounts of sGAG and collagen were homogeneously deposited throughout. This may be due to reduced glucose and oxygen

consumption rates as NP cells naturally reside in a microenvironment with limited nutrient availability. The cell specific response observed in this study may thus be a function of metabolic activity. It is plausible that at a particular oxygen concentration and glucose concentration, NP cells and stem cells possess altering metabolic demands. Agrawal *et al* indicated that oxygen-independent stabilization of HIF-1α, a transcription factor that regulates oxidative metabolism, in NP cells is a metabolic adaptation to a unique microenvironment (Agrawal et al., 2007). Furthermore, it should be noted that these experiments were performed using a cell density of $4x10^6$ cells/ml, which is the typical cell density of native nucleus pulposus tissue. Higher seeding densities that are typically used in tissue engineering investigations would exacerbate the nutrient demands resulting in limited matrix formation. This is an important consideration for IVD regeneration strategies regarding the optimal number of cells that can be injected into the intervertebral disc to elicit a therapeutic response and formation of new tissue. The success of any cell based strategy will therefore be dependent on the state of degeneration and more importantly the microenvironment of the disc that can maintain the viability and support the function of injected cells.

Among several studies that have investigated the effects of IVD-like culture conditions on stem cell survival and differentiation, Weurtz *et al* demonstrated that combining low glucose with high osmolarity (485mOsm) and low pH (6.8) is detrimental to the differentiation of stem cells, with decreased cellular proliferation and collagen and sGAG expression suggesting that the beneficial effects of IVD-like low-glucose culture are not sufficient for promotion of stem cell differentiation when other environmental factors are considered (Wuertz et al., 2008). Of note this study lacks the effect of hypoxia which is known to be a potent regulator of matrix production. Furthermore, it is crucial to determine the response of stem cells to pro-inflammatory cytokines to fully elucidate how these cells

may respond post implantation in a degenerate IVD niche. Culture of stem cells in the presence of IL-1b significantly decreases culture pellet size, and cells produce an ECM with atypical mechanical strength and decreased expression of matrix molecules (Felka et al., 2009).

Considering the highly specialised microenvironment of the central NP, these results indicate that IVD-like low glucose and low oxygen are critical and influential for the survival and biological behaviour of BM stem cells. In this study, for BM constructs, glucose effects were only evident under hypoxic conditions suggesting that low oxygen is an important regulator of matrix production. Furthermore, NP cells and BM stem cells respond differentially to varying environmental conditions due to altered metabolic activity. Under IVD-like microenvironmental conditions, NP cells were found to have a lower glucose consumption rate compared to BM cells and may in fact be more suitable to sustain the harsh microenvironment that exists within the IVD. Such findings may promote and accelerate the development of clinical therapies in demonstrating the suitability of different cell types for targeted regeneration of the IVD.

Acknowledgements

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Figure Legends

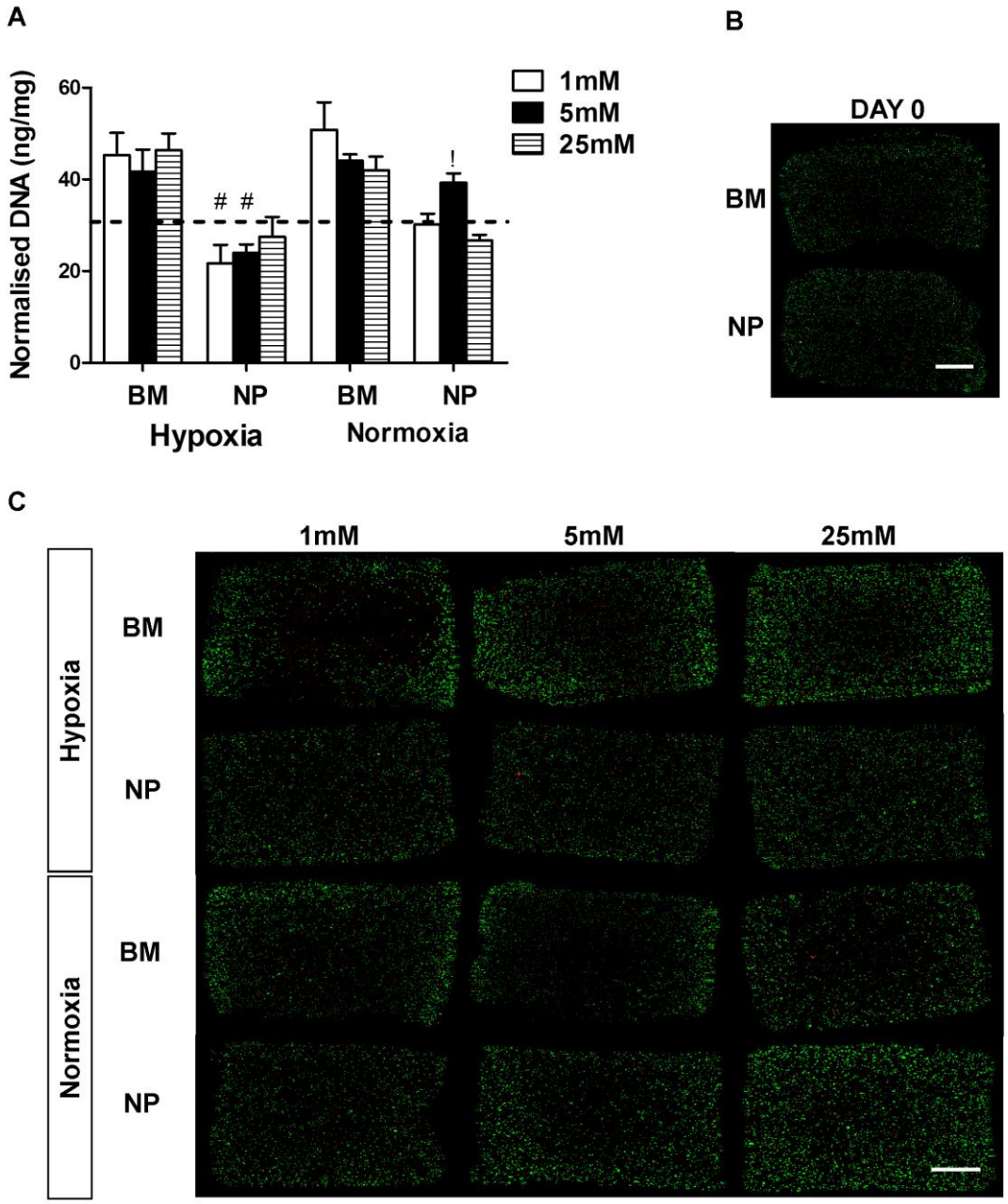

Figure 1 (A) DNA content normalised to wet weight at day 21 for nucleus pulposus (NP) and bone marrow (BM) stem cells maintained under different glucose conditions (1mM, 5mM, 25mM) in hypoxia (5% O_2) or normoxia (20% O_2), # denotes significance compared to normoxia for same cell type, ! denotes significance compared to other glucose condition for same cell type ($p<0.05$); dashed line represents Day 0 DNA content **(B)** Cell viability for NP and BM constructs at Day 0 and Day 21. Scale bar =1mm.

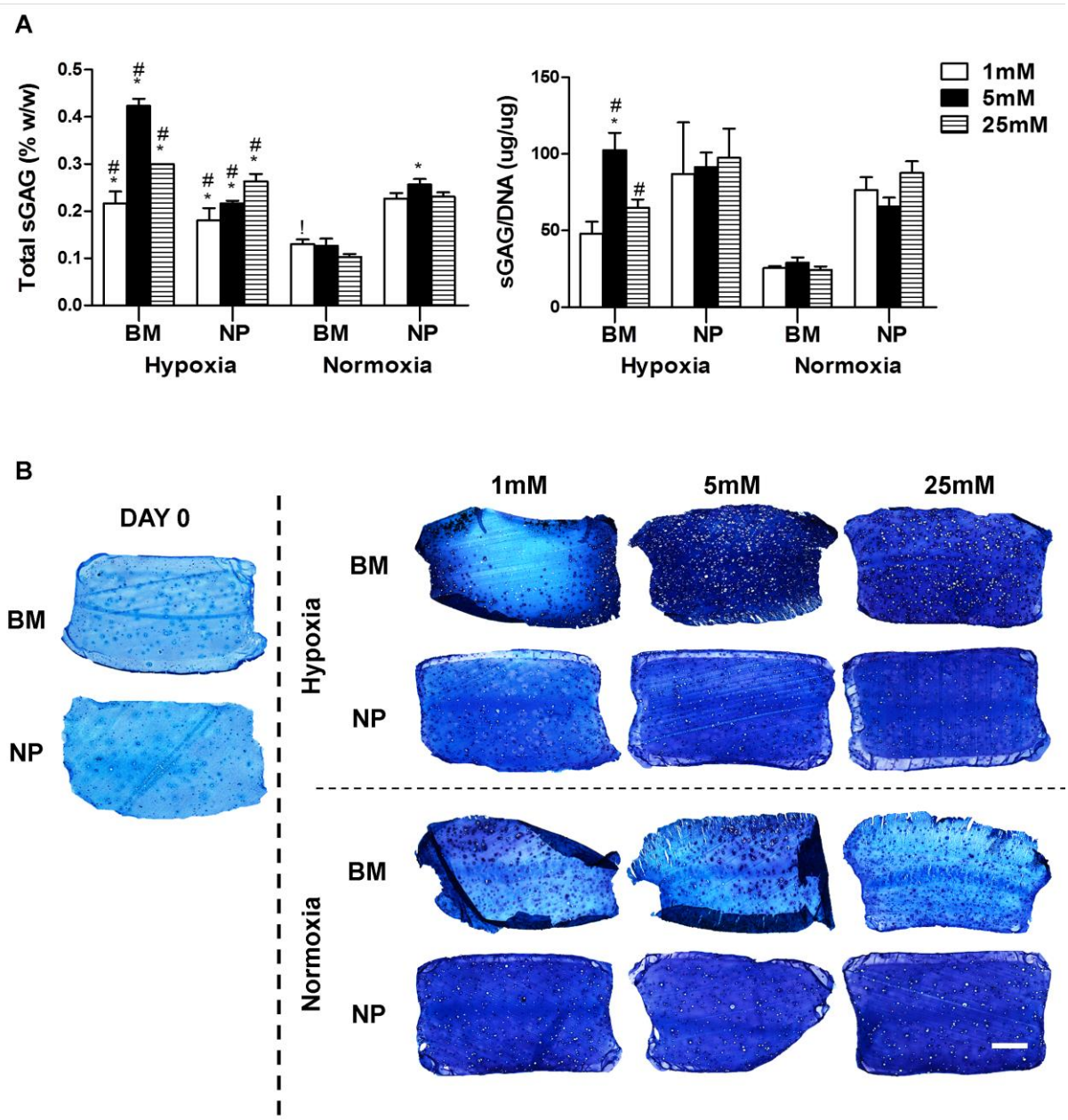

Figure 2 (A) Total sGAG normalized to wet weight and to DNA content at day 21 for nucleus pulposus (NP) and bone marrow (BM) stem cells maintained under different glucose conditions (1mM, 5mM, 25mM) in hypoxia (5% O_2) or normoxia (20% O_2), # denotes significance compared to normoxia for same cell type, ! denotes significance compared to one other glucose condition for same cell type, * denotes significance compared to both other glucose conditions for same cell type ($p<0.05$) **(B)** Histological evaluation with aldehyde fuchsin and alcian blue to identify sGAG at day 0 and day 21; deep blue/purple staining

indicates GAG accumulation and light blue staining indicates residual alginate. Scale bar = 1mm.

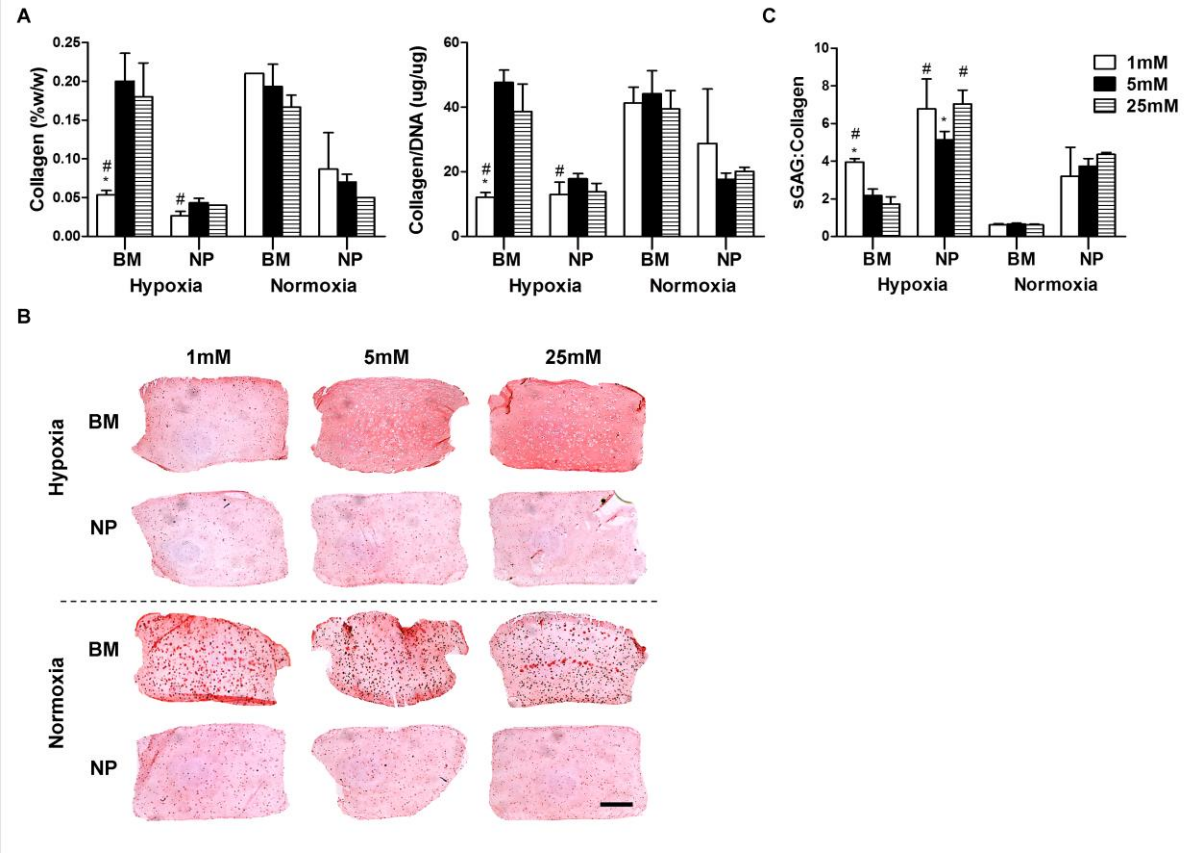

Figure 3 (A) Total Collagen normalized to wet weight and to DNA at day 21 for nucleus pulposus (NP) and bone marrow (BM) stem cells maintained under different glucose conditions (1mM, 5mM, 25mM) in hypoxia (5% O_2) or normoxia (20% O_2), # denotes significance compared to normoxia for same cell type, * denotes significance compared to both other glucose conditions for same cell type ($p<0.05$) **(B)** Histological evaluation with picro-sirius red to identify collagen at day 21. Scale bar = 1mm. **(C)** sGAG:Collagen ratio at day 21; # denotes significance compared to normoxia for same cell type, * denotes significance compared to both other glucose conditions for same cell type ($p<0.05$)

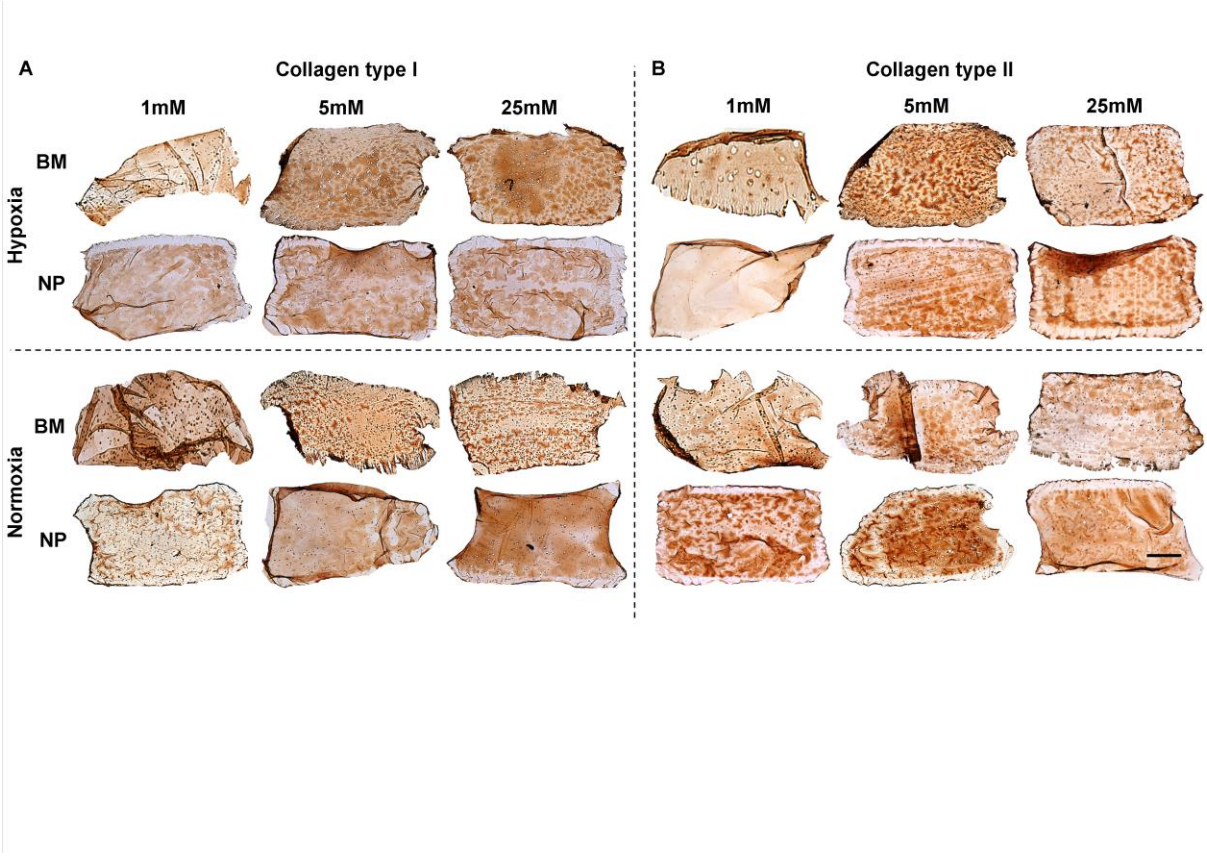

Figure 4 Immunohistochemical evaluation at day 21 for **(A)** Collagen type I **(B)** Collagen type II for nucleus pulposus (NP) and bone marrow (BM) stem cells maintained under different glucose conditions (1mM, 5mM, 25mM) in hypoxia (5% O_2) or normoxia (20% O_2). Scale bar = 1mm.

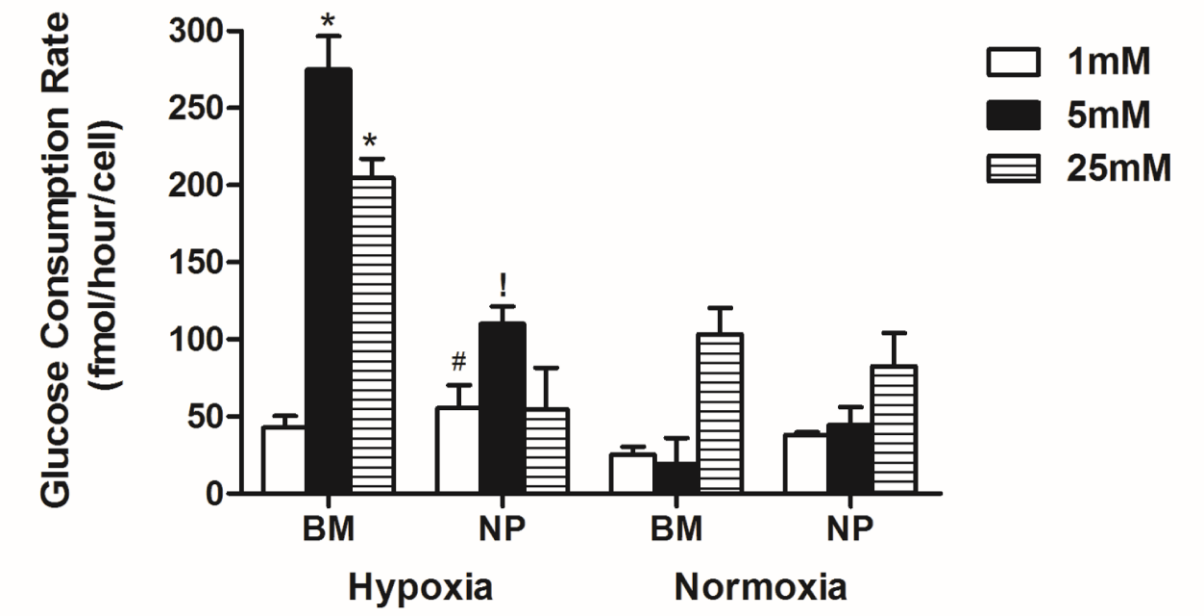

Figure 5 Glucose consumption rate (fmol/hour/cell) at day 21 for nucleus pulposus (NP) and bone marrow (BM) stem cells maintained under different glucose conditions (1mM, 5mM, 25mM) in hypoxia (5% O_2) or normoxia (20% O_2), # denotes significance compared to normoxia for same cell type and glucose condition, * denotes significance compared to all other groups for same glucose condition ($p<0.05$). ! denotes significance for NP in hypoxia compared to all other groups for same glucose condition.

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