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Synergistic Effects of Acidic pH and Pro-Inflammatory Cytokines IL-1β and TNF-α for Cell-based Intervertebral Disc Regeneration

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Abstract

The intervertebral disc (IVD) relies mainly on diffusion through the cartilaginous endplates (CEP) to regulate the nutrient and metabolites exchange, thus creating a challenging microenvironment. Degeneration of the IVD is associated with intradiscal acidification and elevated levels of pro-inflammatory cytokines. However, the synergistic impact of these microenvironmental factors for cell-based therapies remains to be elucidated. The aim of this study was to investigate the effects of low pH and physiological levels of IL-1β and TNF-α on nasal chondrocytes (NCs) and subsequently compare their matrix forming capacity to nucleus pulposus (NP) cells in acidic and inflamed culture conditions. NCs and NP cells were cultured in low glucose and low oxygen at different pH conditions (pH 7.1,

6.8, and 6.5) and supplemented with physiological levels of IL-1β and TNF-α. Results showed that acidosis played a pivotal role in influencing cell viability and matrix accumulation, while inflammatory cytokine supplementation had a minor impact. This study demonstrates that intradiscal pH is a dominant factor in determining cell viability and subsequent cell function when compared to physiologically relevant inflammatory conditions. Moreover, we found that NCs allowed for improved cell viability and more effective NP-like matrix synthesis compared to NP cells, and therefore may represent an alternative and appropriate cell choice for disc regeneration.

Keywords: nasal chondrocytes; disc degeneration; inflammation; microenvironment; nucleus pulposus; cell; spine

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1. Introduction

Low back pain (LBP) is a leading cause of disability, affecting more than 600 million people worldwide [1]. Although it is well established that the causes of LBP are multifactorial and can include mechanical injury [2], genetic predisposition [3] or even lifestyle activities [4], a significant proportion of the cases are associated with intervertebral disc (IVD) degeneration [5, 6]. The IVD is an avascular organ that interfaces the superior and inferior vertebral bodies via the cartilaginous endplates (CEP), regulating the nutrient and waste metabolites path into and out of the IVD, respectively [7]. The microenvironment of a degenerative IVD is considered a hostile niche characterised by large concentration gradients of nutrients and metabolites across its domain, with significantly lower glucose and oxygen concentrations in the centre of the disc, the nucleus pulposus (NP), compared to the periphery, the annulus fibrosus (AF) [8]. Due to the low levels of oxygen in the centre of the IVD, cells residing in the NP rely mainly on anaerobic respiration to create energy, producing lactate as a by-product of glycolysis, and thereby causing acidification of the local

microenvironment. With the onset of disc degeneration CEPs undergo physical changes such as thinning and calcification [9, 10], thereby reducing the bidirectional flow of nutrients and metabolites to and from the NP region causing local lactate accumulation and a consequent decrease in pH [11]. It has been shown that the pH of the disc changes with the degree of degeneration, ranging from pH 7.1 to pH 6.5 in healthy to severely degenerated conditions respectively [12, 13] and that the decrease in the pH in the organ is directly correlated with matrix catabolism [14, 15] and reduced cell viability [16].

The exact mechanisms that trigger degeneration of the IVD are unknown, although it is believed to be mediated by the abnormal production of pro-inflammatory cytokines by NP cells [17, 18]. These cytokines are believed to trigger a series of cellular responses that promote cell senescence, autophagy and apoptosis [19-21]. While a number of pro-inflammatory cytokines have been identified in degenerated discs, tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are believed to play central roles [21-23]. Both IL-1β and TNF-α have been identified to being involved in disc herniation [24], nerve ingrowth [25] and in the upregulation of genes encoding matrix-degrading enzymes [26-28]. Although the correlation of IL-1β and TNF-α with disc degeneration is well established *in vivo*, the response to their *in vitro* supplementation in 2D and 3D cultures has been diverse and often conflicting [19, 23]. One explanation for the differential response observed from *in vitro* experimentation could be the concentration of supplemented cytokines being supraphysiological. In a recent study, Altun *et al.* measured the concentration of IL-1β and TNF-α in degenerated human discs and found their levels to be notably increased in patients affected by acute IVD degeneration, with concentrations in the order of pg/ml [29]. Nevertheless, the concentration of inflammatory cytokines used in the majority of experiments investigating their effects on cells *in vitro* is usually found to be in the order of ng/ml, and significantly higher compared to *in vivo* [30-32].

The role of inflammatory cytokines and environmental pH in IVD degeneration can be linked to many detrimental effects, however it is difficult to determine which of the two has a primary role in disc disease. To fully appreciate the effectiveness of any proposed cell therapy for disc regeneration for clinical use, the regenerative capacity of the cellular component must be examined under physiologically relevant culture conditions.

Identifying an appropriate cell source for disc repair has received significant attention over the last decade, and in particular, the attractiveness of using a patient's own cellular material to avoid complications in relation with immune rejection, supply, ethical and regulatory considerations has been highlighted [33, 34]. Among the various autologous cell sources evaluated in the literature, nasal chondrocytes (NCs) have recently been explored as a potential autologous cell source for cartilage repair [35-37] and may represent a valid alternative for IVD repair strategies [38]. We have previously demonstrated the ability of NCs to remain viable and functional in response to NP-like oxygen and glucose levels [38]. However, the ability of NCs to maintain the same viability and functionality in an inflamed and acidic microenvironment remains to be elucidated.

Hence, the primary objective of this study was to determine whether physiologically relevant concentrations of pro-inflammatory cytokines IL-1β and TNF-α, or an acidic microenvironment impacts cell survival and matrix production of NCs cultured in low glucose and low oxygen conditions. Finally, we compared the functional matrix synthesis capacity of NCs and NP cells in representative physiological conditions of degeneration.

2. Materials and Methods

2.1 Cell isolation and monolayer expansion

Porcine nasal tissue was sourced from a local abattoir and dissected within 24 hours. NC isolation was performed as previously described [39]. Briefly, cartilage from the nasal septum was minced (~2 mm) and digested in serum free Low Glucose–Dulbecco's Modified Eagle

Medium (LG-DMEM) containing penicillin (100 U/mL)–streptomycin (100 µg/ml) (PenStrep) and 3000 U/ml of collagenase type II (Gibco, Invitrogen, Ireland) at a ratio of 10 ml per gram of minced tissue. The digestion of minced tissue was performed under constant rotation for 3 hours at 37°C and subjected to physical agitation using a tissue dissociator (gentleMACS™, Miltenyi Biotech). Cells were separated from tissue residues (40 µm cell strainer) and trypan blue exclusion was used to determine cell yield and viability. NCs were seeded in T-175 flasks ($5x10^3$ cells/cm^2) and expanded to passage two (P2).

NP cells were isolated from the IVDs of porcine spines. Briefly, NP tissues were harvested aseptically and minced. Tissue fragments were placed in T-25 flasks containing LG-DMEM with 10% FBS and PenStrep and cultured in a humidified atmosphere at 37°C and 5% O_2. Once cell migration from the tissue had occurred, flasks were washed to remove debris and NP cells were expanded to 80% confluence and transferred to T-175 flasks ($5x10^3$ cells/cm^2) and expanded to passage two (P2). All expansion cultures contained LG-DMEM supplemented with 10% FBS and 2% PenStrep, and were maintained at 37°C and 5% O_2.

2.2 Preparation of media for experimental culture

All media formulations were prepared from Chemically Defined Medium (CDM) containing LG-DMEM supplemented with 0.25 µg/ml amphotericin B, 2% PenStrep, 100 nM Dexamethasone, 50 µg/ml L-ascorbic acid-2-phosphate, 1% insulin-transferrin-selenium, 4.7 µg/ml linoleic acid, 40 µg/ml L-proline and 1.5 mg/ml bovine serum albumin (BSA). Media was adjusted to pH 7.1, 6.8 and 6.5 by the addition of 400 µl, 450 µl and 500 µl of 3M HCl respectively, and 40 µl of 5M lactic acid (LacA) to 50 ml of CDM to obtain physiological lactate levels (4 mM) normally found in the IVD [11]. Acidic media was subsequently incubated overnight in a humidified atmosphere at 37°C and 5% O_2.

2.3 2D culture under varying pH and inflammatory conditions

Expanded NCs were trypsinised, counted using trypan blue staining and seeded into T-25 flasks at a density of $1x10^4$ cells/cm^2. Cells were allowed to adhere to the culture plastic overnight, and media was changed to pH modified CDM (pH 7.1, 6.8 and 6.5) containing no added cytokines (Control), 125 pg/ml IL-1β, 25 pg/ml TNF-α, or 125 pg/ml IL-1β and 25 pg/ml TNF-α for 7 days at 37°C and 5% O_2. One complete media exchange was performed four days after seeding. Proliferation was assessed in terms of cell counts with trypan blue stain at day 0 and 7, and cell density/morphology was visualised using crystal violet staining.

2.4 Pre-gel fabrication, cell encapsulation and culture

Cells were encapsulated in 3D hydrogels fabricated using a disc extracellular matrix (ECM) derived biomaterial previously developed in our laboratory [40]. Briefly, disc ECM was prepared by solubilizing cryomilled powder in 0.5 M acetic acid (4% w/v) containing pepsin (2.5 mg/ml, Sigma). Solubilised ECM (sECM) and N-hydroxysuccinimide (NHS) functionalised CS (fCS) were combined to yield a final gel composition of 2% sECM-2% fCS. 5% v/v 10x phosphate buffered saline (PBS) was added to the solution and the pH was adjusted to 7.4. A suspension of NCs or NP cells was added to the pre-gel at a cell density of $1x10^6$ cells/ml. Cell seeded pre-gel was cast into cylindrical moulds of 6 mm diameter and incubated at 37°C for 1 hour. Following gelation, the hydrogel constructs were cultured in pH modified CDM (pH 7.1, 6.8 and 6.5) containing no added cytokines (Control), 125 pg/ml IL-1β or 25 pg/ml TNF-α, or a combination of 125 pg/ml IL-1β and 25 pg/ml TNF-α, and incubated for 14 days at 37°C and 5% O_2 . In the second part of the study pH 6.8 groups and groups containing IL-1β or TNF-α only were excluded. Media was changed twice weekly and the supernatant was stored at 4°C for biochemical analysis.

2.5 DNA, sulphated glycosaminoglycan and collagen content

On termination of culture, samples were stored at -80°C until further analysis. Digestion of samples was performed under constant agitation (60°C, 12 hours) with 100 mM sodium phosphate/5mM Na_2EDTA (pH 6.5) and papain enzyme (3.88 U/ml) containing L-cysteine (5mM). Hoechst Bisbenzimide 33258 dye (DNA QF Kit, Sigma-Aldrich) was used for DNA quantification, while the dimethylene blue dye binding assay was used to determine sulphated glycosaminoglycan (sGAG) content. For determining hydroxyproline content, samples were hydrolysed (110°C, 18 hours) with HCL (38%) 18 hours at 110°C in 38% HCl and assayed using chloramine-T [41], and the collagen content determined using a hydroxyproline:collagen ratio of 1:7.69 [42]. Samples of media supernatants were also analysed for both sGAG and collagen content.

2.6 Assessment and analysis of cell viability

A LIVE/DEAD® (Invitrogen) assay was used to determine cell viability. Following a PBS washing step, samples were incubated for 1 hour in phenol free LG-DMEM containing Calcein AM (4 μM) and Ethidium Homodimer 1 (4 μM) (Cambridge Bioscience, UK). Images were captured using an Olympus FV-1000 Point-Scanning Confocal Microscope (515 nm, 615 nm wavelengths). Semi-quantitative analysis of cell viability was determined using ImageJ software (ImageJ, NIH, Bethesda, Maryland).

2.7 Histology and immunohistochemistry

Samples were washed in PBS, treated with 4% paraformaldehyde (4°C, 12 hours), dehydrated in a series of graded alcohols and finally wax embedded. Sections of 8 μm were stained with 1% Alcian Blue 8GX in 0.1 M HCl to assess sGAG deposition. Collagen types I and II were assessed using immunohistochemistry techniques. Sections were treated with

chondroitinase ABC (37°C, 1 hour) (Sigma-Aldrich), and non-specific sites were blocked using 5% BSA. Collagen type I (Abcam) and collagen type II (Santa Cruz) primary antibodies incubated at 4°C overnight. The secondary antibody (Anti-Mouse igG biotin conjugate, Sigma-Aldrich) was applied for 1 hour followed by incubation (45 minutes) with ABC reagent (Vectastain PK-400, Vector Labs, UK). DAB peroxidase (Vector Labs, UK) was used as a developer.

2.8 Statistical analysis

GraphPad Prism (Ver. 7) was used for presentation of graphical data (mean ± standard deviation) and statistical analysis (two-way ANOVA), with significance accepted at a level of $p<0.05$. N is used to represent the number of biological donors and n to represent the technical replicates for each experiment performed.

3. Results

3.1. pH and inflammatory cytokine effects on NC in 2D culture

There was an increase in the number of viable cells after 7 days of 2D culture for pH 7.1 control and pH 7.1 IL-1β groups compared to day 0 levels. Conversely, TNF-α or of IL-1β+TNF-α groups did not exhibit significant changes compared to day 0 samples in terms of cell number (Fig 1.A). As expected, there was a decrease in the number of viable cells with increasing acidity. However, it is unclear whether lower cell numbers were as a result of diminished proliferation, increased cell death, or a combination of both. Cellular density as assessed through crystal violet staining confirmed the biochemical results with no noticeable differences in cell morphology observed for the different groups investigated (Fig 1.B).

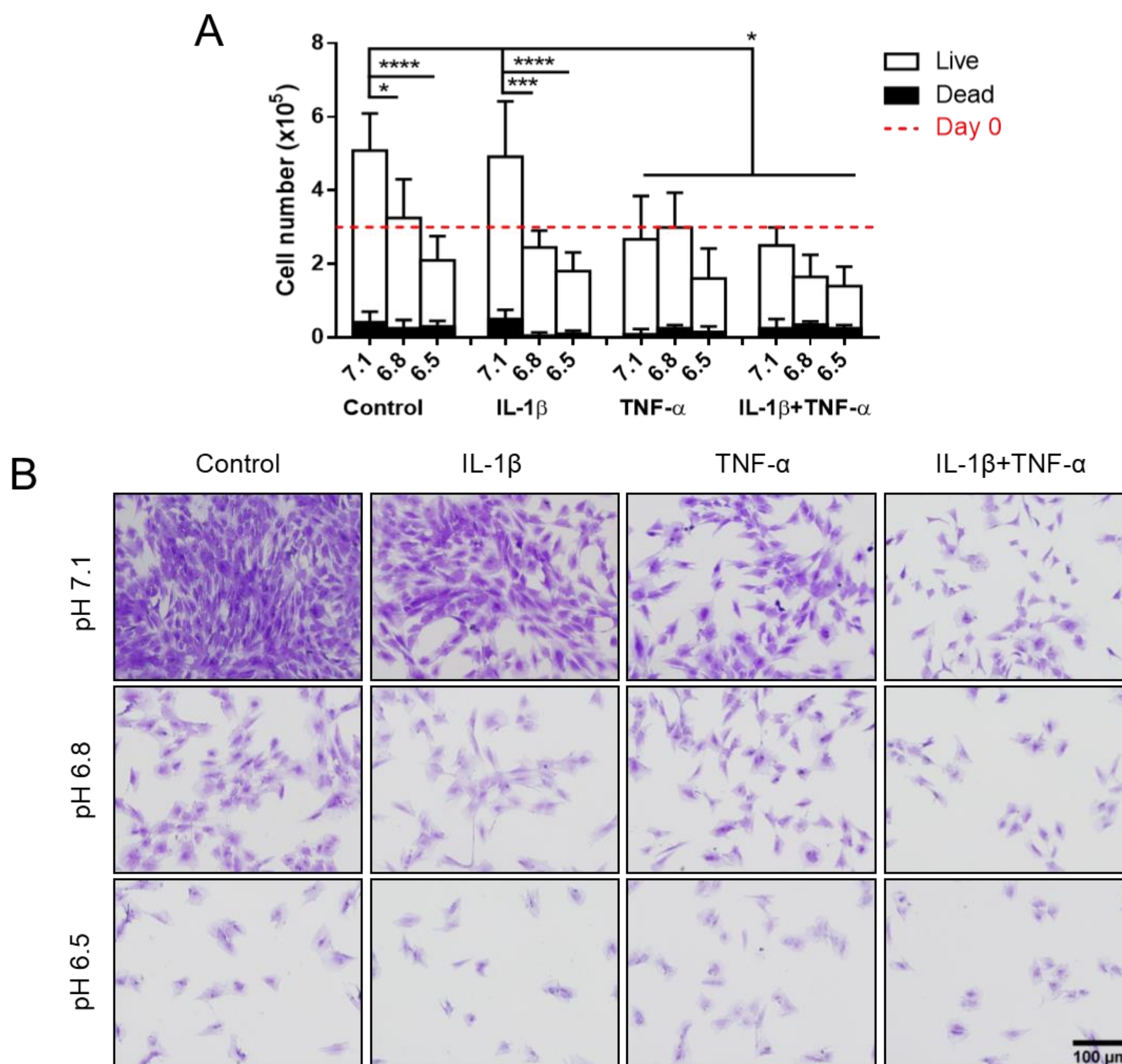


Figure 1 Effects of pH and inflammatory cytokines on NC proliferative capacity. (A) Viable cell count at day 7 for NCs on 2D tissue culture plastic cultured in pH modified media (pH 7.1, 6.8 and 6.5) either unsupplemented (control) or supplemented with IL1-β, TNF-α or a combination of both. *($p<0.05$), ***($p<0.001$) and ****($p<0.0001$) indicate significant differences between groups. N=1 donor, n=3 samples. (B) Crystal violet staining of NCs at day 7. Scale bar is 100 µm.

3.2. Acidic pH and inflammatory cytokines negatively impact cell viability in 3D hydrogels

When cultured in 3D hydrogels, NCs appeared to be affected by culture conditions in a similar fashion to those observed for 2D culture. Semi-quantitative analysis based on Live/Dead images of hydrogels at day 14 showed a correlation between culture conditions

and cell viability with acidic pH, and on a smaller scale the presence of inflammatory cytokines, negatively influencing cell viability (Fig 2.A,B). At pH 6.5, supplementation with IL-1β+TNF-α had a more detrimental effect on cell viability than supplementation with single cytokines. However, this result was not confirmed by DNA quantification, with no significant differences observed among sub-groups at pH 6.5 and 6.8 (Fig 2.C). Nevertheless, DNA content was found to be lower for groups cultured in acidic pH media, irrespective of the presence of inflammatory cytokines, with the lowest DNA content observed for all pH 6.5 media formulations.

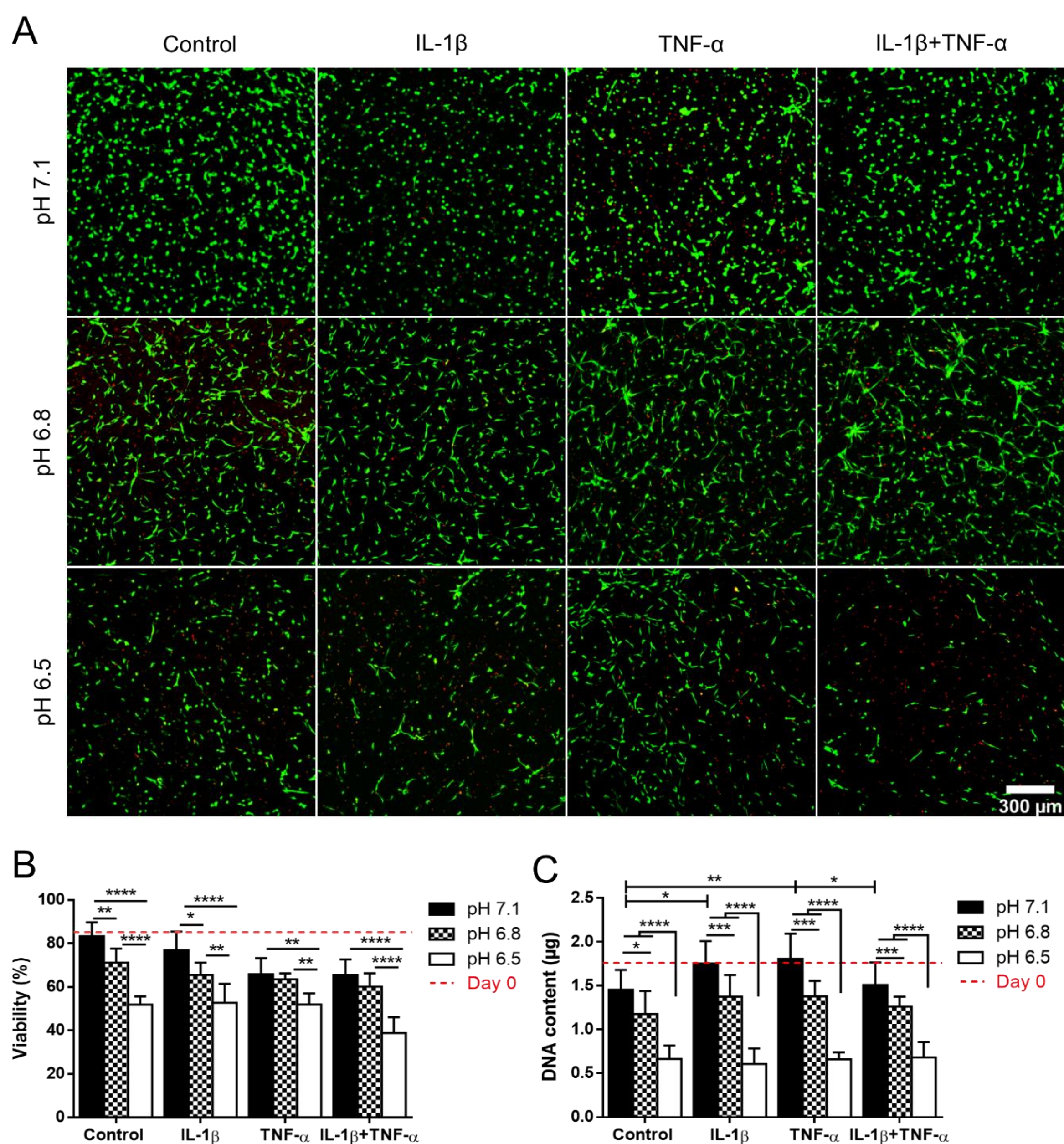


Figure 2 (A) Live/Dead images of NCs at day 14 cultured in 3D hydrogels in pH modified media (pH 7.1, 6.8 and 6.5) either unsupplemented (control) or supplemented with IL1-β and TNF-α or a combination of both. Scale bar is 300 µm. (B) Cell viability (%). (C) DNA content (µg) of hydrogels at day 14. *($p<0.05$), **($p<0.01$), ***($p<0.001$) and ****($p<0.0001$) indicate significant differences between groups. N=3 donors, n=3 samples.

3.3. Matrix synthesis in response to different pH and inflammatory cytokine conditions

Control ECM hydrogels (no inflammatory cytokines) cultured in media at pH 7.1 exhibited the highest collagen accumulation which was diminished with increasing acidic media formulations. Similar collagen levels were observed for all inflammatory cytokine supplemented groups at all pH levels. At pH 7.1 a noticeable decrease in collagen deposition was observed for IL-1β+TNF-α supplemented formulations compared to control (Fig 3.A). The majority of collagen detected at day 14 was found to have been released into the culture media (Fig 3.B). Immunohistochemical staining of collagen types I and II did not reveal obvious differences in pericellular collagen deposition across the groups (Fig 3.C).

Acidity was observed to decrease the ability of cells to synthesise sGAGs, while inflammatory cytokine supplementation did not have any noticeable effect (Fig 4.A). All pH 7.1 groups exhibited a sGAG to collagen ratio that was significantly higher compared to pH 6.8 and pH 6.5 groups. Interestingly, significant differences in sGAG:collagen ratios were noted among groups cultured at pH 7.1 supplemented with different combinations of inflammatory cytokines. Groups cultured at pH 7.1 supplemented with IL-1β and IL-1β+TNF-α were significantly higher than the pH 7.1 control group (Fig 4.B), due to reduced collagen deposition. Histological staining was found to corroborate the sGAG biochemical findings, with more intense staining at pH 7.1 at day 14 (Fig 4.C).

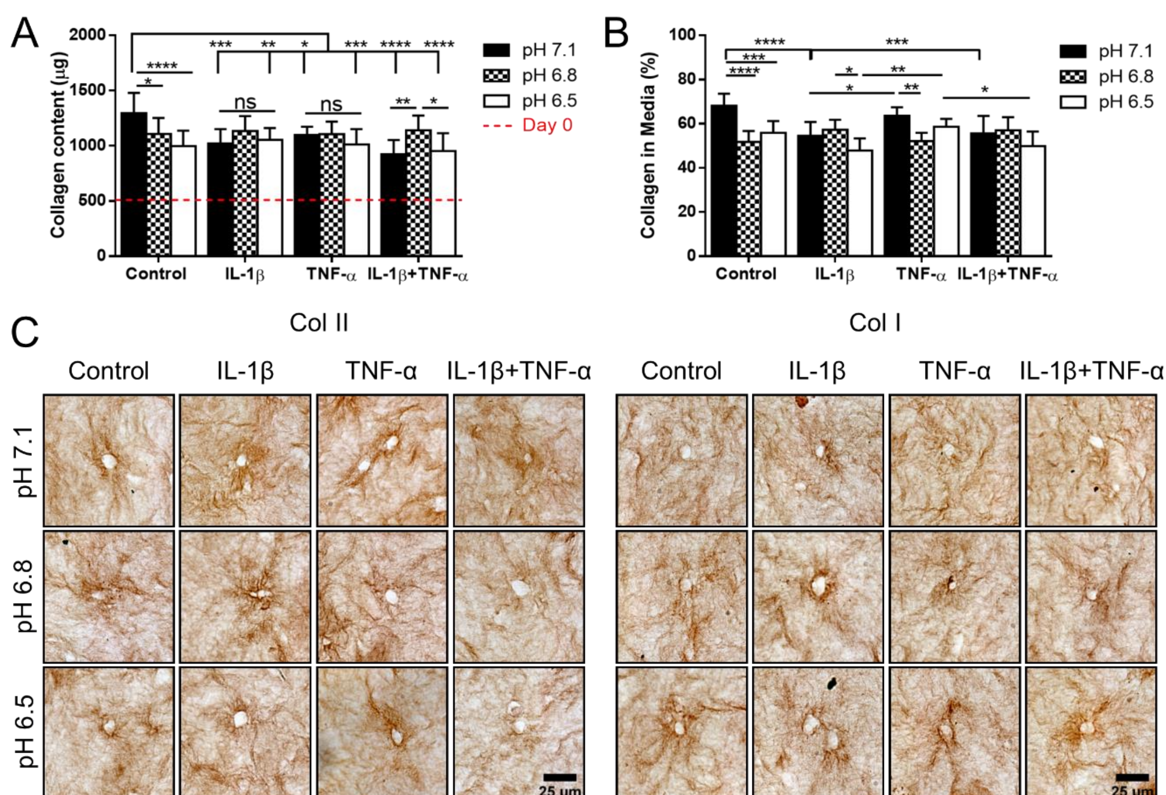


Figure 3 (A) Total collagen content (μg) in hydrogels and media at day 14 cultured in pH modified media (pH 7.1, 6.8 and 6.5) either unsupplemented (control) or supplemented with IL1-β and TNF-α or a combination of both. (B) Collagen detected in culture media (%). The values are expressed as a percentage of the total amount of collagen measured at day 14. *($p<0.05$), **($p<0.01$), ***($p<0.001$) and ****($p<0.0001$) indicate significant differences between groups. N=3 donors, n=3 samples. (C) Collagen type II and collagen type I immunohistochemical staining. Scale bar is 25μm.

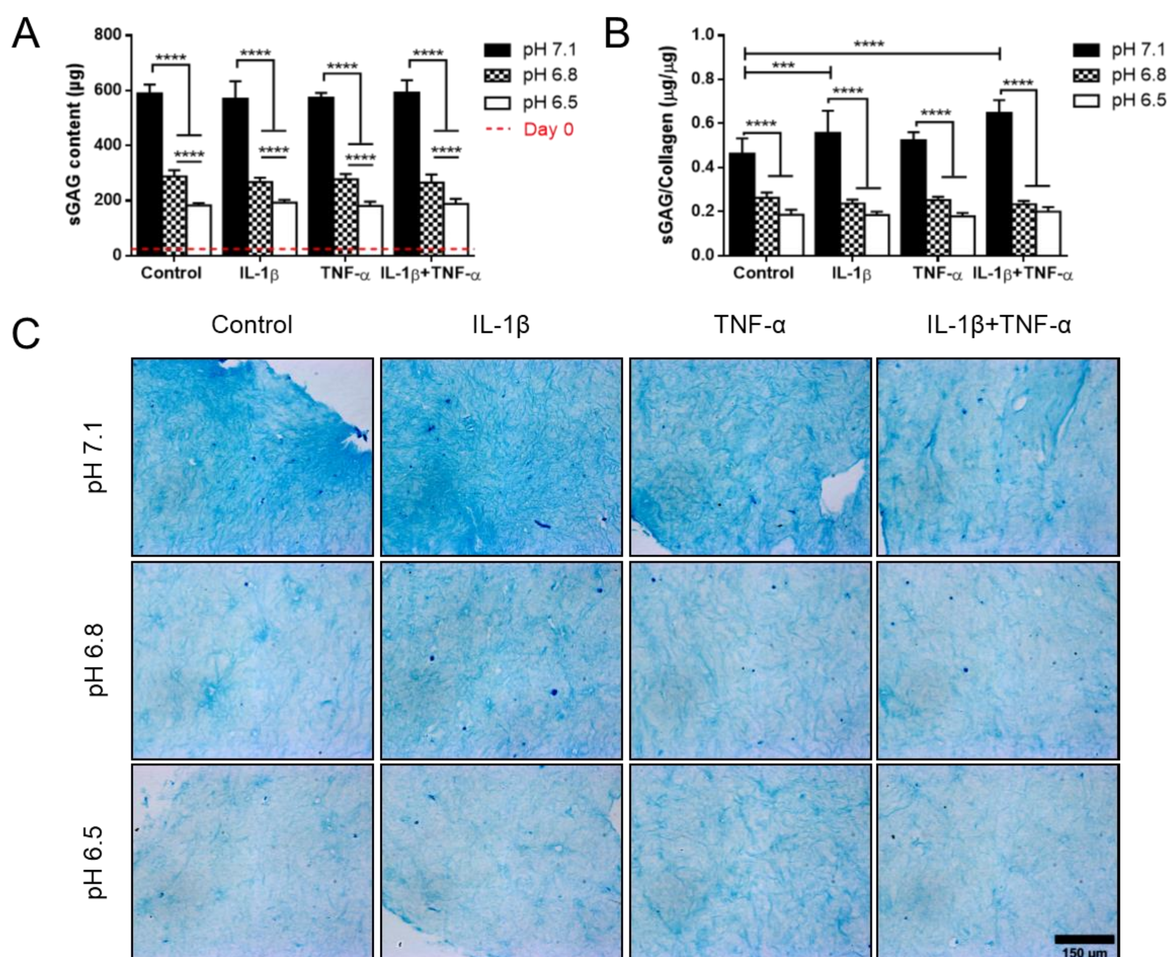


Figure 4 (A) sGAG content (µg) in samples (hydrogels+culture media) at day 14 cultured in pH modified media (pH 7.1, 6.8 and 6.5) either unsupplemented (control) or supplemented with IL1-β and TNF-α or a combination of both. Dashed line represents day 0 sGAG content. (B) sGAG:Collagen ratio. Both sGAG and collagen were calculated as respective amounts produced over 14 days by subtracting day 0 content from day 14. ***(p<0.001) and ****(p<0.0001) indicate significant differences between groups. N=3 donors, n=3 samples. (C) Alcian blue histological evaluation for sGAG accumulation in hydrogels at day 14. Scale bar is 150µm.

3.4. Acidic pH and inflammatory cytokines have a greater impact on NP cell viability and proliferation

Motivated by our previous results, the effect of acidic media culture in the presence of physiologically relevant inflammatory cytokine concentrations was subsequently assessed

for NCs and NP cells in 3D hydrogel culture. pH media groups were reduced to pH 7.1 and pH 6.5 to simulate healthy and acute degeneration conditions respectively, with or without the combined supplementation of both IL-1β+TNF-α. Live/dead analysis revealed reduced viability for NP cells compared to NCs at pH 7.1 and pH 6.5 in both control and IL-1β+TNF-α supplemented conditions. Acidity was again observed to be the primary factor responsible for cell death, as shown by the significant decrease in cell viability among cells cultured in acidic media compared to the controls (Fig 5A,B). Quantification of DNA further confirmed the detrimental effect of acidic pH on NCs and NP cells in control and inflamed conditions (Fig 5C).

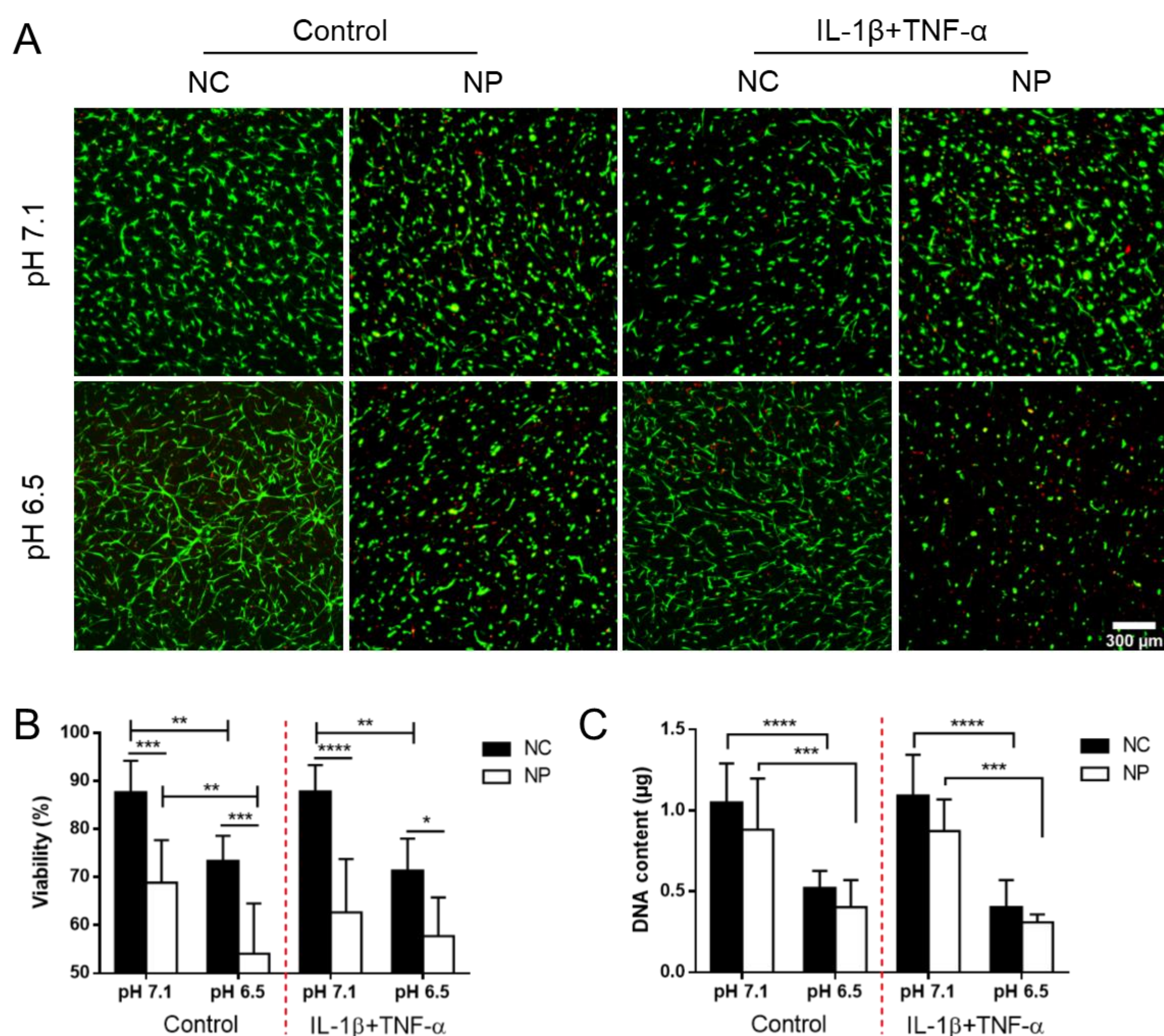


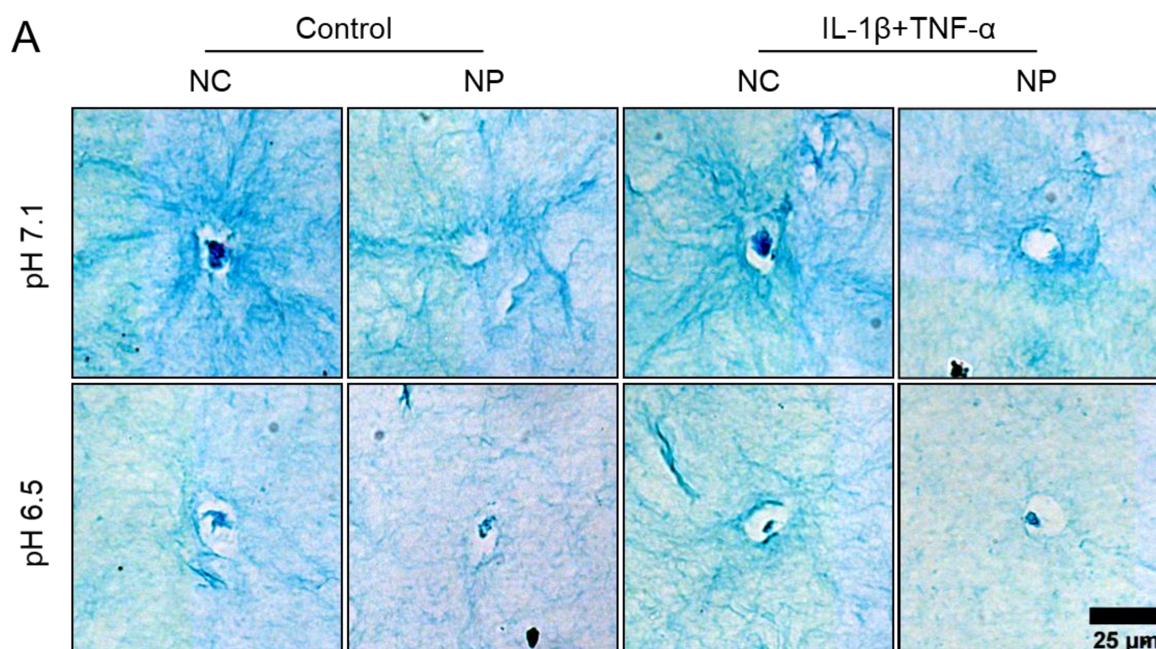
Figure 5 (A) Live/Dead imaging at day 14 of NCs and NP cells in 3D hydrogel culture at different pH (7.1 and 6.5) and inflammatory cytokine (IL-1β+TNF-α)

supplementation conditions. Scale bar is 300μm. (B) Cell viability (%). (C) DNA content (μg) of hydrogels at day 14. *($p<0.05$), **($p<0.01$), ***($p<0.001$) and ****($p<0.0001$) indicate significant differences between groups. N=3 donors, n=3 samples.

3.5. NCs can secrete higher amounts of key matrix components compared to NP cells in a degenerated disc-like environment

Acidity was also observed to have a negative impact on sGAG synthesis. Intense localised sGAG deposition was observed for NCs cultured in pH 7.1 media (control and inflamed conditions), with less intense staining observed for NP cells. At pH 6.5, both NCs and NP cells displayed diminished sGAG staining. There were no appreciable differences in staining intensity between control and inflamed conditions at either pH levels. Biochemical results supported these histological findings, demonstrating that NCs cultured in pH 7.1 media were able to synthesise significantly higher amounts of sGAGs compared to all other groups by day 14. There was no significant impact of culturing in IL-1β+TNF-α supplemented media for either cell type (Fig 6.A, B).

In terms of collagen content, cells cultured in pH 7.1 control media showed enhanced collagen production compared to pH 6.5 control media. However, no differences were detected in collagen synthesis when cells were cultured in IL-1β+TNF-α supplemented media (Fig 7.A). Moreover, a similar pattern was found for sGAG:collagen ratios among groups, with NCs exhibiting a higher ratio than NP cells which was higher for groups cultured in pH 7.1 media than pH 6.5. Interestingly, NCs cultured in pH 7.1 media supplemented with IL-1β+TNF-α exhibited a sGAG:collagen ratio significantly higher than in pH 7.1 control media (Fig 7.B), due to diminished collagen deposition. Immunohistochemical staining demonstrated higher levels of collagen type II at pH 7.1 which was diminished at pH 6.5. Culturing with IL-1β+TNF-α did not appear to affect the type of collagen being deposited (Fig 7.C).



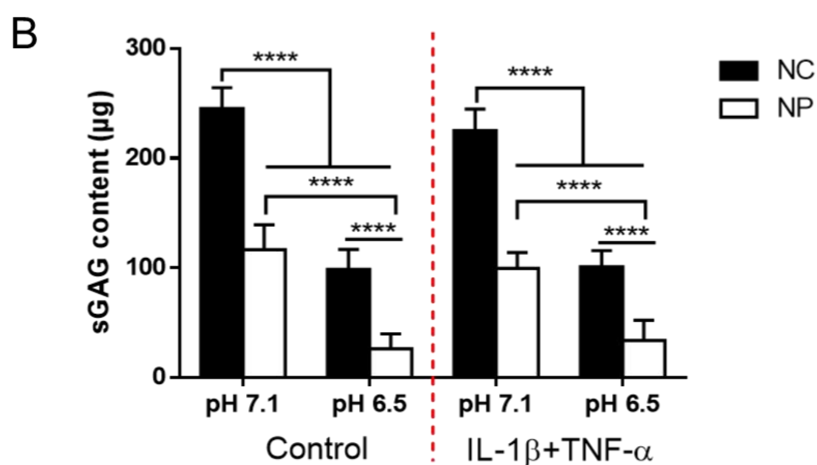


Figure 6 (A) Histological evaluation of hydrogels with alcian blue to identify sGAG at day 14 of NCs and NP cells in 3D hydrogel culture at different pH (7.1 and 6.5) and inflammatory cytokine (IL-1β+TNF-α) supplementation conditions. Scale bar is 25µm. (B) sGAG (µg) produced by NCs and NP cells in 3D hydrogels. Values were calculated by subtracting day 0 content from day 14. ****($p<0.0001$) indicates significant differences between groups. N=3 donors, n=3 samples.

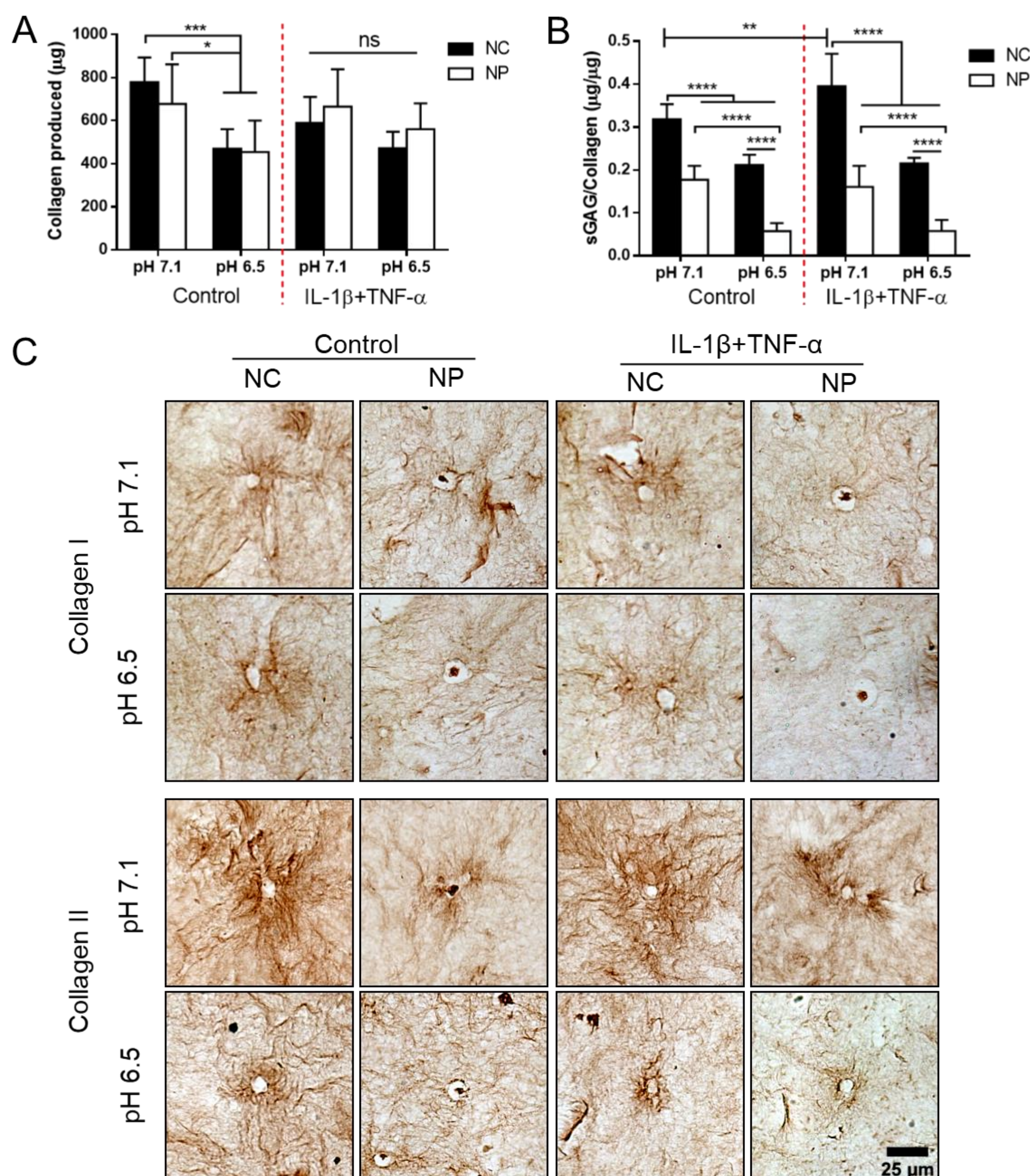


Figure 7 (A) Total collagen (µg) produced in hydrogels containing NCs and NP cells in 3D hydrogel culture at different pH (7.1 and 6.5) and inflammatory cytokine (IL-1β+TNF-α) supplementation conditions. (B) sGAG:Collagen ratio. Both quantities were calculated as respective amounts produced over 14 days by subtracting day 0 content from day 14. *($p<0.05$), **($p<0.01$), ***($p<0.001$) and ****($p<0.0001$) indicate significant differences between groups. N=3 donors, n=3 samples. (C) Collagen type I and collagen type II immunohistochemical staining. Scale bar is 25µm.

4. Discussion

The IVD is a large avascular organ relying mainly on diffusion of nutrients through the CEPs for its energy supply. During disc degeneration calcification of the CEPs hinders the availability of nutrients to cells residing in the centre of the disc and the removal of metabolic by-products of glycolysis, thereby causing acidification of the cellular microenvironment and a shift to an accelerated catabolic state [14, 15]. Moreover, the degenerated disc consists of a wide panel of pro-inflammatory cytokines [22, 29, 43], further contributing to the creation of an extremely challenging biochemical microenvironment. For successful translation of cell-based therapies for disc regeneration, it is imperative that cells remain viable post injection, and function effectively in a challenging microenvironment [8, 33, 34].

In the first part of this study we investigated the impact of the pro-inflammatory cytokines IL-1β and TNF-α, in combination with low pH media in 2D and 3D hydrogel culture with NCs. It was found that acidic pH affected cell viability and proliferation to a much greater extent in comparison to IL-1β and TNF-α supplementation. The detrimental effect of acidic pH was also observed in relation to a significant reduction in matrix synthesis in 3D culture, whereas IL-1β and TNF-α supplementation, either alone or in combination, appeared to have no influence on sGAG synthesis and only a marginal influence on collagen production when NCs were cultured at pH 7.1.

Surprisingly, we found that cytokine supplementation had only a marginal influence on cell viability and matrix synthesis. Cellular response to culture in inflammatory conditions has been extensively studied in the last two decades. However, there is still no consensus on the specific molecular mechanisms that are triggered or enhanced by the presence of pro-inflammatory cytokines such as IL-1β and TNF-α, possibly due to the variability in culture conditions and consequently in the results obtained. Some studies have found strong evidence of IL-1β being the key regulator of matrix degradation processes in NP cells [28, 44], and identified the role of TNF-α more likely to be associated with nerve root irritation rather than

influencing matrix catabolism [23]. In contrast, a number of other studies have either not found major differences between the effects of IL-1β and TNF-α supplementation on NP cells [27, 45], or even reconsidered the central role that was attributed to IL-1β in the process of matrix catabolism [19, 43]. It is possible to find analogous discrepancies in similar studies on articular chondrocytes (ACs): whereby some authors have judged the contributions of both IL-1β and TNF-α as equal [46], others have found evidence of TNF-α having a predominant influence on cell death [47] and decreased matrix synthesis [48]. Nevertheless, in contrast with our results, the common denominator of all these findings is that the presence of pro-inflammatory cytokines, either alone or in combination, deeply affects cell fate and gene expression. It must be noted, however, that in all these previous studies cells have been exposed to concentrations of IL-1β and TNF-α of the order of ng/ml, which is supraphysiological even for pathological conditions such as the more acute stages of degeneration of the IVD. In a recent study from Altun et al., biopsy specimens from surgically excised discs from patients diagnosed with acute disc disease were shown to contain amounts of IL-1β and TNF-α in the range of 113-135 pg/ml and 9-26 pg/ml respectively [29], which are more than three orders of magnitude lower than the concentrations used in previous experiments. Our results suggest that when cell cultures are supplemented with IL-1β and TNF-α at physiologically relevant concentrations, cellular responses are not as marked as described in previous experiments and that these cytokines affect cell viability and matrix synthesis less dramatically compared to environmental pH.

The effects of low pH on cell viability have been explored in the last two decades on different cell sources such as bone marrow stem cells (BMSCs) [49], adipose-derived mesenchymal stem cells (ADMSCs) [50], ACs [51, 52] and NP cells [53]. Results from these studies are in line with our findings, demonstrating a correlation between increasing environmental acidity and decreased cell viability. One possible explanation for this could be that culture in acidic media induces the activation of acid-sensing ion channels (ASICs),

which are extracellular pH sensors whose activation has been found to be responsible for acidosis-mediated apoptosis [54]. The expression of this family of receptors has been observed in several cell types, including disc cells and ACs [51, 54-56]. Although the expression of ASICs has not yet been examined for chondrocytes extracted specifically from the nasal septum, it is reasonable to assume that their response to acidic environments would be driven by the same biochemical mechanisms that drives the response of chondrocytes derived from articular cartilage. Activation of ASICs has been shown to be enhanced under inflammatory conditions, in particular in the presence of IL-1β, resulting in increased cell death [57]. However, such a correlation was not observed in our results, which showed no significant differences in cell viability following IL-1β supplementation in 2D or 3D cultures.

Acidic pH also had a significant impact on the ability of NCs to synthesise and deposit de novo matrix. We observed a reduction in the synthesis of sGAGs compared to controls (pH 7.1) in NCs cultured in media at pH 6.8 and 6.5, which is not simply due to compromised cell viability. It has previously been reported that cell metabolism can be severely inhibited by low pH [58, 59]. This is due to phosphofructokinases, the rate-limiting enzymes on glycolysis, being sensitive to pH and in particular inhibited by acidosis [60]. As a consequence, cells have less energy available and processes tightly linked to energy consumption such as sGAG synthesis are inhibited or impeded [61]. Interestingly, although we did observe a higher amount of collagen being produced at pH 7.1 without inflammatory factors, the detrimental impact of acidic conditions was less pronounced in comparison to sGAG synthesis, suggesting that the molecular mechanisms regulating collagen synthesis are less dependent on energy availability. A similar finding was reported in a study from Nishida et al., where a decrease in energy production in hypoxic conditions promoted hypertrophy in ACs with a decrease in aggrecan and an increase in collagen expression [62].

A second objective of this study was to directly compare the ability of NCs and NP cells to synthesise NP-like matrix in culture conditions mimicking the degenerated disc niche (low

glucose, low oxygen, low pH in the presence of pro-inflammatory cytokines), with the aim of determining an appropriate cell source for disc repair strategies. We found that NCs, although still negatively affected by low pH, maintained higher cell viability and synthesised appropriate matrix components such as sGAGs and collagens in a more appropriate ratio than NP cells. In comparison, NP cells exhibited diminished cell viability at pH 7.1, representative of a healthy disc, which also resulted in a reduction in the accumulation of sGAGs when compared to NCs. This correlates with results from Razaq et al, where the expression profile of bovine AC and NP cells cultured in acidic conditions was compared, demonstrating a significantly lower expression of matrix related genes with increasing acidity for NP cells [53]. Importantly, the sGAG:collagen ratio was considerably higher in NCs compared to NP cells for all culture conditions. A high sGAG:collagen ratio may be considered an appropriate parameter for the identification of NP-like tissue type, as it represents a tissue rich in sGAGs, replicating the composition typical of healthy NP [63].

In conclusion, the results of the present study demonstrate that among the plethora of characteristics that render the degenerated NP an extremely hostile niche, environmental pH was found to have the greatest impact on cell viability and matrix production. While NP cells appeared to be unaffected by the presence of inflammatory cytokines in the physiological range, low pH had a greater impact on viability and matrix synthesis, suggesting they may be a less effective cell type compared to NCs for cell-based NP regeneration. Although it is clear that environmental acidity is an important factor to consider in the context of disc regeneration, to the best of the authors knowledge, there are no clinically feasible approaches to neutralising or augmenting the intradiscal pH. A possible approach may involve injecting pH buffering biomaterials into the disc space. This may have some short-term benefit and create a permissible microenvironment to facilitate regeneration by transplanted cells, but the longevity of such an approach would need to be established. In parallel, techniques to non-invasively measure intradiscal pH in vivo would be required in order to tailor or adjust the

treatment according to the severity of the degenerated state. Recent work has proposed a promising clinical MRI approach to detect pH changes associated with IVD degeneration in a swine model [64]. Overall, the presence of the key inflammatory cytokines IL-1β and TNF-α, that have previously been identified in human discs did not have any appreciable impact when supplemented in physiologically relevant concentrations. Therefore, developing imaging modalities to identify and characterise intradiscal pH *in vivo* in combination with pH buffering biomaterials may provide for an effective strategy to appropriately select and treat patients with cell-based therapies.

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