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Effect of nutrient deprivation on the viability of intervertebral disc cells

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Abstract There is evidence that a fall in nutrient supply leads to disc degeneration but little understanding of the effects of nutrient deprivation on the physiology of disc cells which govern the composition of the disc. We examined the effects of changes in glucose and oxygen concentration and pH on the viability and metabolism of cells from bovine nucleus pulposus. Cells isolated from bovine discs and embedded in alginate beads were cultured under oxygen and glucose concentrations from zero to physiological levels and maintained at pH 7.4, pH 6.7, or pH 6.2 for up to 3 days. Interactions between nutrient concentrations were examined in relation to cell viability and lactic acid production. Cell viability was significantly reduced in the absence of glucose, with or without oxygen. Disc cells survived at 0% oxygen, provided that glucose was present, as seen previously. Cell viability decreased if the medium

was acidic, more so when combined with low glucose concentrations. The rate of lactic acid production also fell as the pH became acidic and after 24 h or more at low glucose concentrations, but it did not appear to vary with oxygen concentration under the culture conditions used here. Glucose, rather than oxygen, appears to be the nutrient critical for maintaining disc cell viability. However, in an avascular tissue such as the disc, it is unlikely that glucose deprivation will occur alone; it will almost certainly correlate with a fall in oxygen concentration and pH. These results indicate that the combined nutrient and metabolite environment, rather than concentrations of any single nutrient, should be considered when studying cellular physiology in the disc.

Keywords Extracellular pH · Glucose · Nucleus pulposus cells · Oxygen concentration · Chondrocyte

Introduction

The intervertebral disc is avascular: almost the entire disc receives its nutritional supply from blood vessels in the vertebral body that terminate at the vertebral endplate [36]. Nutrients must then diffuse from these blood vessels across the endplate and through the matrix to reach the cells of the disc. This route can be up to 7 mm or 8 mm long in adult human lumbar discs [6]. Metabolites and other cellular products must also travel out of the disc via this route to reach the circulatory system.

Cellular energy metabolism in the disc is dominated by anaerobic glycolysis. Disc cells thus consume glucose and produce lactic acid to generate energy in the form of ATP [18]; there is also a small but significant consumption of oxygen [5, 18]. Steep gradients in the concentrations of these nutrients and metabolites arise, even in normal healthy discs [18], because rates of nutrient consumption are high compared to rates of diffusion through the matrix [34, 35]. As a result of these gradients, cells in the centre of the disc exist at low concentrations of oxygen and glucose and a high concentration of lactic acid (hence an acidic pH). Oxygen concentrations as low as 1% [3, 18]

and pH levels as low as pH 5.7 [10] have been measured in the centres of some discs.

A fall in nutrient supply to the disc has long been implicated in the development of disc degeneration [6]. Factors which limit the blood supply to the disc, (such as smoking [4] and atherosclerosis [23]), and hence decrease the supply of nutrients to these cells are associated with disc degeneration. Calcification of the endplate, as seen in ageing and some disc disorders [28, 32], leads to a fall in endplate permeability and thus hinders the transport of nutrients into the disc [1, 7, 16, 33, 37]. Loss of endplate permeability is also associated with disc degeneration [29].

Despite this evidence of a link between reduced nutrient supply and disc degeneration, there is at present little understanding of the cellular consequences of nutrient failure. There is however some evidence that low concentrations of nutrients or acidic pH levels are detrimental to the activity of disc cells: rates of synthesis of protein and proteoglycan in disc explants have been found to decrease markedly *in vitro* at either low oxygen concentrations or low pH [21, 30, 31]. If the extracellular nutritional environment becomes too extreme, it may even lead to cell death: *in vitro* studies have found cell viability to be sensitive to either acid pH or low glucose [19, 31]. In these experiments, nutrient and metabolite concentrations were varied independently. However, in the avascular disc, local concentrations of oxygen, glucose, and lactic acid are determined by the balance between solute transport and cellular metabolism: thus changes in the concentrations of these solutes can not occur independently *in vivo*.

It is at present not known how conditions in the centre of the disc (low oxygen, low glucose, and acidic pH) affect cell viability, nor which nutritional and metabolic parameters in particular are critical. In this work, we investigated cell viability and metabolism in relation to pH and to concentrations of glucose and oxygen, both in combination and alone.

We chose to study nucleus pulposus cells, as they will be the cells most affected by alterations in nutritional supply to the disc. Bovine nucleus pulposus cells are phenotypically similar to those of the adult human nucleus [11, 20] and were thus used in this study. We isolated disc cells for these experiments to remove the gradients in nutrient concentrations which develop in intact tissue sections [18]. We used a low ratio of cells to incubation medium, to maintain constant extracellular concentrations during incubation. We assayed for lactic acid concentration as a general marker of cellular metabolism in cartilaginous cells [24] and also to investigate the Pasteur effect (i.e. the increase in the rate of glycolysis as the oxygen supply falls). This has been reported to occur in slices of disc tissue [18, 21] and would further lower glucose concentrations and increase the concentration of lactic acid (and thus lower pH) in the hypoxic centre of the disc.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) with 25 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 1000 mg/L glucose, and pyroxidine ('standard' DMEM), DMEM without glucose or phenol red ('no-glucose' DMEM), penicillin G sodium, streptomycin sulfate, and amphotericin B were obtained from Invitrogen, Paisley, Scotland, UK. Alginate acid (sodium salt) was obtained from Fluka Biochemika, Gillingham, Dorset, UK. Calcium chloride and ethylenediamine tetraacetate (EDTA) were obtained from BDH, Poole, Dorset, UK. Bleach was obtained from Proctor and Gamble, Weybridge, Surrey, UK. Unless stated otherwise, all other materials were obtained from Sigma-Aldrich, Poole, Dorset, UK. Both types of DMEM were made to approximately 380 mOsm by sodium chloride addition, and antibiotics and antimycotics added at the following concentrations: penicillin G 100 U/ml, streptomycin 100 µg/ml, and amphotericin B 0.25 µg/ml.

Methods

Cells were isolated from fresh bovine caudal discs (within 2 h of slaughter) by collagenase digestion (30 mg collagenase/tail) in standard DMEM (0.5% weight/volume) for 19 h (37°C, 100% humidity, 5% CO₂, 21% O₂, balance N₂) as described previously [22, 31]. Digested tissue and medium were filtered with a Falcon 40-µm filter to remove nondigested fragments (Fahrenheit, Milton Keynes, UK) and washed three times by centrifugation (5 min at 1118 *G*) and resuspension. Trypan blue exclusion was used to determine cell viability; only cell preparations with viability of >98% were used.

Cells were diluted to 8 million cells/ml in alginate solution (1.2% w/v sodium alginate, 0.9% w/v NaCl, 0.2-µm sterile-filtered), mixed by slow pipetting, and transferred to a sterile syringe. This concentration of cells was found to have no adverse effect on cell viability or metabolism for up to 1 week (Adam Meir, personal communication). Alginate beads were formed by allowing drops of solution to fall from the syringe needle (27 gauge) into calcium chloride (102 mM, 0.2 µm sterile-filtered), where the alginate polymerised on contact [9, 27]. Beads were rinsed twice in sodium chloride (0.9% w/v, 0.2 µm sterile-filtered), then incubated in standard DMEM at a concentration of 0.5 million cells/ml for 24 h.

After initial incubation, beads were transferred to the wells of Falcon sterile plates (Fahrenheit). No-glucose DMEM was made to 0 mM, 0.5 mM, or 5 mM glucose and aliquots of each solution adjusted to pH of 7.4, 6.7, or 6.2. Since serum contains glucose, no serum was used in these short-term experiments. This solution was added to the wells at a concentration of 0.2 million cells/ml DMEM. For each value of glucose and pH, one plate was cultured at 37°C and 100% humidity in an incubator with gas containing 21% O₂, 5% CO₂, and the balance N₂. Another plate was placed in a sealed vessel (tested at low oxygen concentrations and found to be airtight) and flushed exhaustively with 5% CO₂ and 95% N₂. The concentration of oxygen in the medium of culture plate wells inside the vessel had been previously tested and was found to fall to zero. This experimental protocol gave 18 different combinations of pH and glucose and oxygen concentrations; each was performed in triplicate.

At 5 h, 24 h, and 48 h, a bead was removed from each well and assayed for cell viability. Alginate beads were dissolved in a 1:3 mix (w/v) of alginate and citrate buffer (1.6% w/v sodium citrate, 1.8% w/v EDTA, 0.9% w/v sodium chloride) [27]. This was left for 5–10 min, with gentle agitation, and weighed to determine total volume. Trypan blue exclusion was then used to determine cell viability.

Medium from each well was sampled at the same time points (4×10 µL), for lactic acid concentration as measured by a standard

colorimetric enzymatic procedure (Sigma procedure no. 735). Absorbance was calibrated against a standard curve produced from known concentrations of lactic acid in unconditioned no-glucose DMEM. At conclusion of the experiment, a sample of medium was streaked onto sterile nutrient agar (1.5%) and incubated to check that infection was not present (24 h, 37°C).

Statistical analysis

Data were analysed using analysis of variance software (SPSS, Chicago, Ill., USA).

Results

Cell viability

After 5 h of incubation, no change in cell viability was observed in any sample. However, after 24 h a significant fall in cell viability was seen in samples incubated with no glucose and at pH 6.2 or 6.7, both with and without oxygen, as shown in Fig. 1 ($P<0.01$). The interaction between low pH and no glucose was found to be significant ($P<0.01$), i.e. a combination of these two factors caused more cell death than would be expected from their additive effects

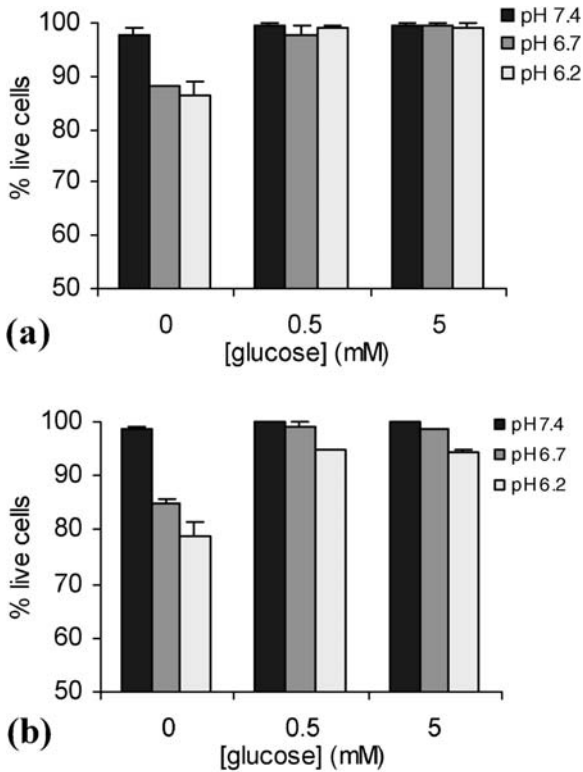


Fig. 1 The effects of pH and glucose concentration on the viability of disc nucleus pulposus cells cultured in alginate beads at 0% (a) and 21% oxygen (b) after 24-h incubation ($n=3$ for each bar). Results are given as mean+SEM. $P<0.01$

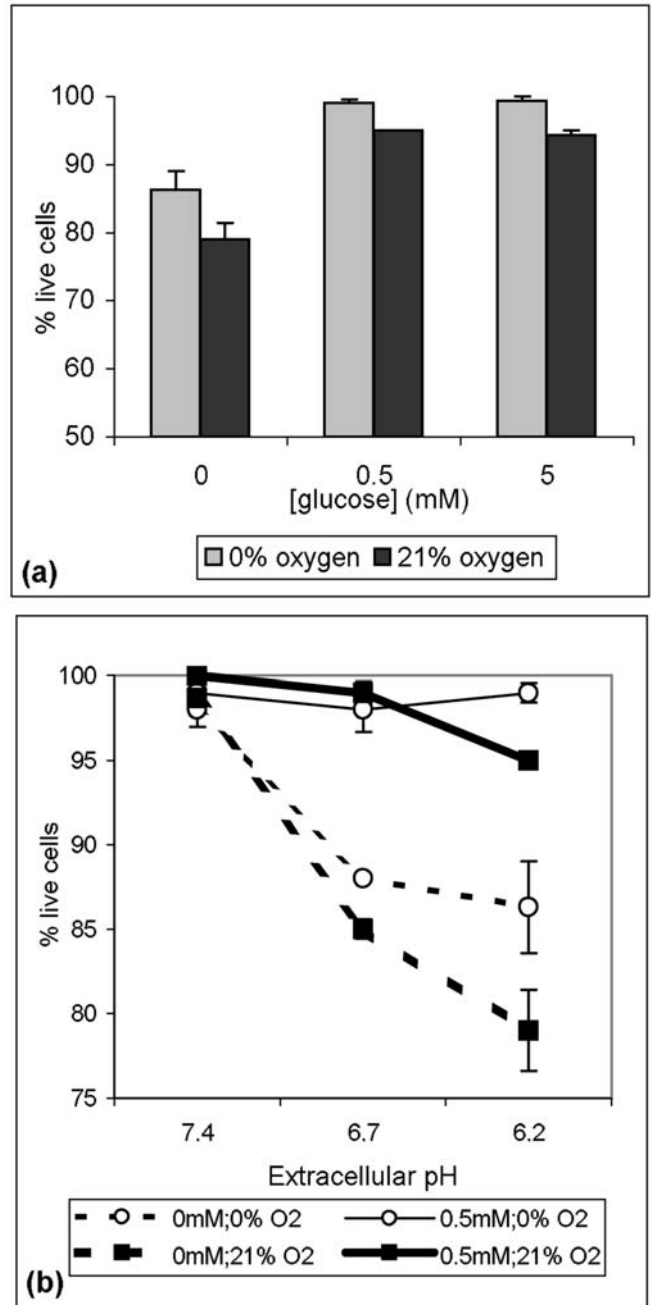


Fig. 2 The effects of the presence of oxygen on cell viability (a) at pH 6.2 and (b) low glucose concentrations and various pH levels after 24 h incubation ($n=3$ for each bar). Results are given as mean±SEM $P<0.01$

acting individually. After 48 h, cell death was observed in all samples at 0 mM glucose, including those at pH 7.4.

In cells incubated under extreme conditions (i.e. no glucose and acidic pH), cell death was seen to occur more extensively in samples incubated at 21% oxygen than at 0% oxygen (Fig. 2). At 21% oxygen (atmospheric concen-

tration), a fall in cell viability was observed at pH 6.2 at all glucose concentrations ($P<0.01$), more so than in cells incubated under hypoxia (at the same glucose concentrations and pH). After 48 h, viability had also fallen with neutral pH in the absence of glucose ($P<0.01$), more severely so at 21% oxygen than 0% ($P<0.01$).

Lactic acid production

Lactic acid production did not differ significantly between different glucose concentrations after 5 h of incubation, probably because the cells with low glucose levels were using glycogen stores accumulated during their previous

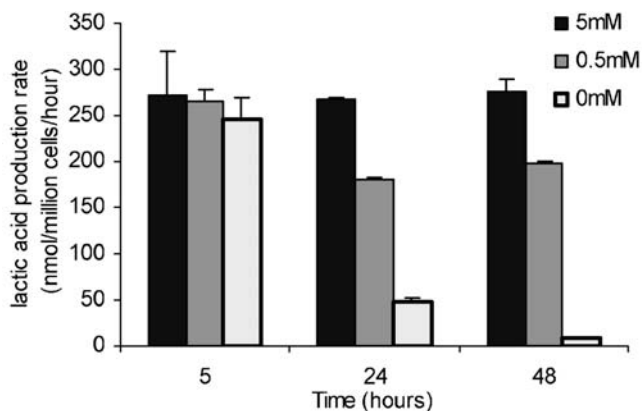


Fig. 3 Lactic acid production at various glucose concentrations after 5 h, 24 h, and 48 h of culture at 21% oxygen and pH 7.4 ($n=3$ for each bar). Results are given as mean+SEM. $P<0.01$

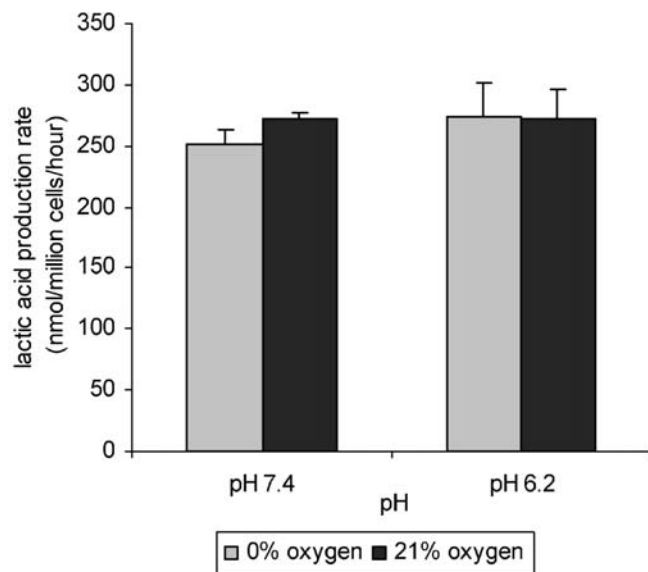


Fig. 4 Lactic acid production at 5 mM glucose and neutral and low pH at 0% or 21% oxygen levels ($n=6$ for each bar). Results are given as mean±SEM. $P<0.01$

incubation with 5 mM glucose. After 24 h and 48 h of incubation, lactic acid production was significantly reduced in cells incubated in 0.5 mM of glucose and further reduced with 0 mM, compared to lactic acid production by cells incubated in 5 mM of glucose ($P<0.01$) (Fig. 3). This suggests that glycogen stores adapt to new extracellular concentrations of glucose after 24-h incubation. At 0 mM glucose, lactic acid production had almost ceased after 48 h (under which conditions cell viability had fallen to 50%).

There was no significant or consistent effect of the presence or absence of oxygen on lactic acid production (Fig. 4) at any pH or glucose concentration; i.e. there was no evidence of any Pasteur effect.

There was a significant inhibitory effect of low pH on metabolism ($P<0.01$); however, this effect disappeared when cells were incubated without oxygen (Fig. 5). This fall in lactic acid production at low pH also disappeared in the absence of glucose; however, cell death had begun to occur in these cells, so these results are not definitive.

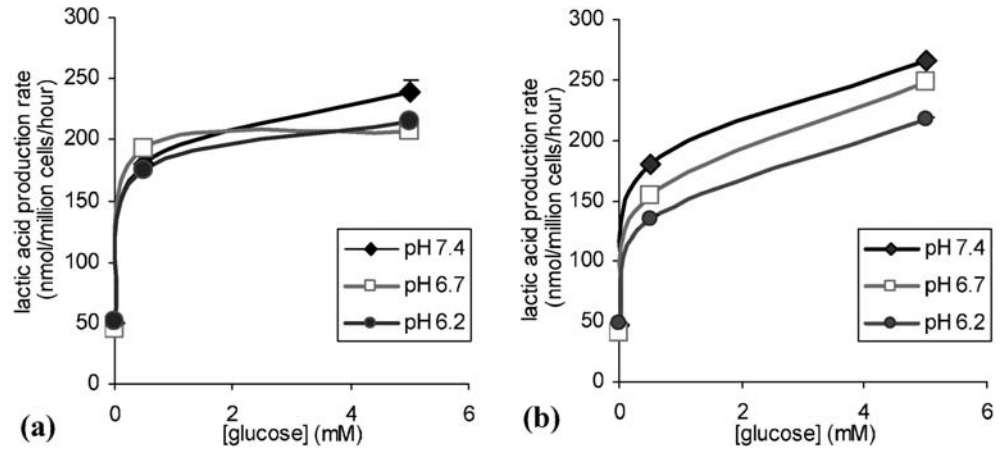
Discussion

A number of studies have investigated the effects of hypoxia on cellular metabolism in the disc and other cartilages and have found that it leads to a dramatic fall in the synthesis of matrix macromolecules [13, 14, 15, 18, 21, 38]. However, in ischaemia or in an avascular tissue such as the disc, it is unlikely that hypoxia will occur alone; it will almost certainly correspond with a fall in glucose concentrations and pH. In this study, we investigated these factors together and indeed saw an interaction between nutrient and metabolite concentrations and their effects on cell viability.

We examined the effect on cell viability of acidic pH and low oxygen and glucose concentrations. These results show a fall in cell viability in bovine nucleus pulposus cells with low glucose but not low oxygen (Fig. 1, Fig. 2), in agreement with other studies [19, 31]. It thus appears that glucose may be the limiting nutrient for the survival of disc cells, rather than oxygen, as has previously been proposed [35]. Glucose has also been proposed as the limiting nutrient for cell viability in tumour spheroids [8]. Low pH however also appears to lead to cell death (Fig. 1, Fig. 2), in agreement with other studies [19, 31]. Here we saw an interaction between glucose levels and extracellular pH; cell viability at low pH and in medium with no glucose was lower than would be expected from these two factors acting individually (Fig. 1, Fig. 2).

Hypoxia, rather than causing cell death, appeared to be protective under the extreme conditions of acidic pH and no glucose (Fig. 2): the fall in viability was lower in cells incubated at 0% oxygen than at 21% oxygen with the same pH and glucose concentrations. An increase in survival under hypoxia (as opposed to atmospheric levels) in cells incubated at low glucose has also been seen in tumour cells and fibroblasts [12].

Fig. 5 Lactic acid production by disc cells cultured at various pH levels and glucose concentrations after 24 h under (a) 0% oxygen or (b) 21% oxygen. Results are given as mean \pm SEM. $P < 0.01$ ($n = 3$ for each bar)



Lactic acid production, as a marker of metabolism, was seen to fall at low glucose concentrations (Fig. 3). This was as expected, since glucose is the main substrate for glycolysis and hence lactic acid production. The latter was also reduced at low pH, as found previously [31]. We did not see a consistent or significant effect of oxygen tension on lactic acid production, i.e. no Pasteur effect (Fig. 4). A positive Pasteur effect has been reported in experiments on samples of disc tissue cultured in DMEM [18, 21]. However, lactic acid production has previously been reported to be unaffected by oxygen tension when culturing isolated cells in DMEM: the Pasteur effect was seen in neither bovine nucleus pulposus cells nor articular chondrocytes cultured in DMEM [5, 31]. In contrast, a negative Pasteur effect was reported for isolated bovine nucleus cells [5] and articular cartilage slices [24] cultured in a solution of salts plus glucose. Thus it appears that both the cellular environment (isolated from the matrix vs in situ) and DMEM vs a simple salt solution may play a part in the presence or absence of the Pasteur effect.

Unlike salt solutions, DMEM contains amino acids and vitamins. In a recent study investigating the effects of both low glucose and hypoxia on the survival of fibroblasts and tumour cells, it was found that a protective effect was conveyed by amino acids [12]. Esumi et al. hypothesise that a switch from glucose to amino acids as the main energy source occurs under 'starvation' conditions (i.e. low oxygen and low glucose) [12]. Thus there may be a difference in the metabolic pathways used at low oxygen between cells incubated in DMEM, where amino acids are present, and those incubated in salt solutions. Another possible reason for the apparent absence of a Pasteur effect in DMEM could be the presence of oxidants in the medium. Lee et al. have recently shown that, in cartilage slices, the fall in lactic acid production (i.e. the negative Pasteur effect) does not occur in the presence of alternative oxidants [25]. Oxidants present in DMEM (for example riboflavin or sodium pyruvate) could possibly act in a similar manner and maintain glycolysis at low oxygen tension. What-

ever the mechanism, the composition of the culture medium appears involved in the effect of oxygen tension on lactic acid production, i.e. in the presence or absence of the Pasteur effect.

It is not clear from this work or from the literature what the effect of hypoxia on lactic acid production is in vivo. A negative Pasteur effect could occur, as found experimentally using isolated cells in a salts-plus-glucose medium [5]; no Pasteur effect could occur, as seen in isolated cells in DMEM (Fig. 4) [31], or a positive Pasteur could occur, as reported in tissue slices incubated in DMEM [18, 21]. More importantly, the advantage to the disc of increased glycolysis under hypoxia is questionable for several reasons.

Firstly, in vitro experiments that showed a positive Pasteur effect (in the presence of an adequate glucose supply) still reported a reduction in the synthesis of matrix macromolecules under hypoxia [21]. This reduction was similar to that when no Pasteur effect was observed [31]. Matrix synthesis rates are closely coupled to intracellular ATP levels [2, 26]; it thus appears that the increase in glycolysis under hypoxia (the positive Pasteur effect) is insufficient to prevent a fall in intracellular ATP concentrations and hence can not prevent a fall in matrix synthesis. Therefore, the advantage of the Pasteur effect is not apparent in this case. Secondly, a positive Pasteur effect may even be harmful. As extracellular oxygen concentration falls towards the centre of the disc, so will the glucose concentration [34]. An increase in the rate of glycolysis under hypoxic conditions may then lead to depletion of an already limited supply of glucose. If an absence of glucose does indeed cause cell death (as results seen here and by others indicate [19]), increasing the rate of glycolysis in cells may lead to more widespread cell death. Thirdly, if a positive Pasteur effect occurs, lactic acid production will increase in the hypoxic regions in the centre of the disc, further lowering the pH [10]. This fall in pH will in turn decrease the production of lactic acid (Fig. 5) [31] and thus tend to offset the increase caused by the positive

Pasteur effect. The fall in pH may also cause a decrease in cell viability (Fig. 1), which will be detrimental to the disc.

Conclusions

Nutrient supply is thought to be a factor limiting cell density in the avascular disc: discs over a range of vertebrate species show an inverse relationship between cell density and disc thickness [17]. Evidence also exists that a fall in nutrient supply is a pathway to disc degeneration. Low concentrations of oxygen and low values of pH have pre-

viously been shown to have adverse effects on rates of matrix synthesis in the disc [19, 30, 31]. Here we found that disc cells could not survive without an adequate supply of glucose and that low pH also limited cell viability. Both glucose concentration and pH levels decrease towards the centre of the disc and fall more steeply at higher cell densities or if endplate permeability is reduced [34]. The results of this study therefore support the idea that a fall in nutrient supply is one pathway to disc degeneration.

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