EQUINE CHONDROCYTE METABOLISM UNDER HYPOXIA

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by

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ABSTRACT

Equine chondrocyte metabolism under hypoxia

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The avascular nature of articular cartilage limits oxygen supply within the tissue. Cartilage, therefore, is under physiological hypoxic conditions. Oxygenation gradients are estimated from 10% at the surface to 1% at the deepest layer. Nonetheless, chondrocytes have been reported to be able to survive and are well-adapted to such an environment. We hypothesised that low oxygen tensions favour chondrocyte metabolism based on their nature and literature reviews.

Our study focused on investigation of three major functions of chondrocytes, which are glucose transportation, catabolism and anabolism under a low oxygen environment compared to atmospheric condition. A key regulator of cellular responses to hypoxia, HIF, was also included in this study. However, due to a limitation of the detection techniques, we were not able to identify the expression of oxygen-sensitive unit, HIF-1α in equine chondrocytes.

Study of glucose transportation showed increases of glucose uptake and expression of GLUT1 protein (FACS analysis) and mRNA of equine chondrocytes under hypoxia indicating their metabolic adaptation to low oxygen environment. We also demonstrated downregulation of GLUT1 mRNA in pathologic cartilage such as found in OA and OCD which may indicate compromised glucose transportation leading to failure of tissue repair.

Hypoxia had only a very small effect on cytokine-induced cartilage degradation. Although we found reduced amounts of TNF-stimulated MMP-13 activation under hypoxia, GAG and collagen releases were not affected by oxygen levels.

Hypoxia showed variable effects on cartilage matrix synthesis, depending upon different joint sources of chondrocytes and the number of their passage. This may indicate interaction of hypoxia and such factors that needed to be considered in cartilage engineering study.

Overall, our study clearly showed metabolic adaptation of chondrocytes under different oxygen levels. Hypoxia, while having no obvious effect on cartilage degradation showed contradictory results in cartilage neo-genesis suggesting complex factor involvement.

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ABBREVIATIONS

v/v	volume/volume
w/v	weight/volume
x g	times of gravity
g	gram
mg	milligram
µg	microgram
l	litre
ml	millilitre
µl	microlitre
M	molar
mM	millimolar
µM	micromolar
mm	millimetre
µm	micrometre
nm	nanometre
cm^2	square centimetre
min	minute
pmol	picomol
°C	degree Celsius
2-D	two dimension
3-D	three dimension
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
Ala	alanine
ANOVA	analysis of variance
APMA	*p*-aminophenylmercuric acetate
APS	ammonium persulphate
ATP	Adenosine tri-phosphate
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium
bHLH	basic helix-loop-helix
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
Cha	L-cyclohexylalanine
Ci	curie
CNBr	cyanogen bromide
CO_2	carbon dioxide
$CoCl_2$	cobalt chloride
COMP	cartilage oligomeric matrix protein
CS	chondroitin sulphate
$CuSO_4$	copper sulphate
DAB	dimethylamino benzaldehyde

DMEM	Dulbecco's modified Eagles medium
DMMB	1,9-dimethylmethylene blue dye binding assay
DNA	deoxyribonucleic acid
dNTP	nucleotide
Dpa	3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ESI-MS/MS	electrospray ionization mass spectrometry/mass spectrometry
FACIT	fibril associated collagens with interrupted triple helices
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FSC	forward light scatter
G1	globular domain 1
GAG	glycosaminoglycan
GLUT	glucose transporter
Gly	glycine
H_2O	water
HBS	HIF-1 binding site
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF	hypoxia inducible factor
His	histidine
HRE	hypoxic response elements
IGD	interglobular domain
IGF	insulin-like growth factor
IgG	immunoglobulin G
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
ITS	insulin-transferin-selenium
KCl	potassium chloride
kDa	kilodalton
KS	keratan sulphate
LPS	lipopolysaccharide
LTRF	lateral trochlea ridge of the femur
MFI	mean fluorescence intensity
MFS	major facilitator superfamily
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSDB	mass spectrometry protein sequence database
MT-MMP	membrane type matrix metalloproteinase
Na_2CO_3	sodium carbonate

NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na-K tartrate	sodium potassium tartrate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
Nva	L-norvaline
O_2	oxygen
OA	osteoarthritis
OCD	osteochondritis dissecans
OD	optical density
ODD	oxygen-dependent degradation
OSM	oncostatin M
PAS	Per-Arnt-Sim
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PHD	prolyl-4 hydroxylase
Pro	proline
PVDF	polyvinylidene fluoride
pVHL	von Hippel-Lindau tumour suppressor protein
qPCR	quantitative (real-time) polymerase chain reaction
Q-ToF	Quadrupole-Time of Flight
RIN	RNA integrity number
RIPA	radioimmuno precipitation assay
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SOX	SRY (sex determining region Y)-box
SSC	side light scatter
TE buffer	Tris-EDTA buffer
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TIMP	tissue inhibitors of metalloproteinases
TNF	tumour necrosis factor
V	volt

Chapter I

GENERAL INTRODUCTION

Joint structure

Diarthrodial joints consist of several structures, including the articulating surfaces of bones covered by articular cartilage, the synovial membrane, the fibrous capsule, a cavity containing synovial fluid, and the supportive ligaments (Fig 1.1). The synovial structure is designed to facilitate joint movement and load bearing. Surrounding ligaments stabilise the joint and prevent over-flexion or -extension. The capsule composes of two layers, the outer membrane which is a fibrous layer, defining the joint boundary and the inner layer or synovial membrane, which contains and is responsible for synthesis of synovial fluid. The synovial fluid lubricates the joint to prevent inflammation and pain during movement and also allows diffusion of nutritive substances from the highly vascularised synovial membrane to joint tissues (Simkin and Pizzorno 1974).

Articular Cartilage

Articular cartilage is the connective tissue lining the ends of bones within diarthrodial joints. The resident cells within this tissue, the chondrocytes, secrete and organise a unique extracellular matrix, which is glassy or

hyaline in appearance and is deformable, facilitating a smooth gliding movement and load distribution.

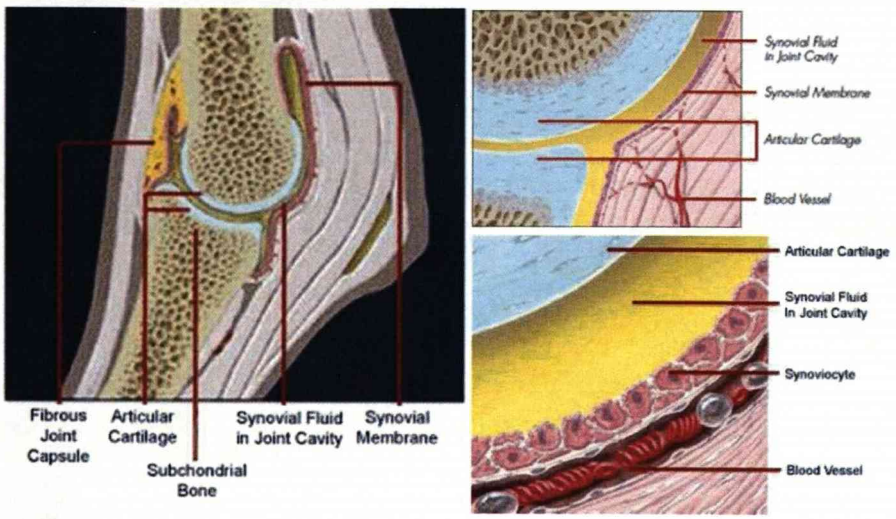

Fig 1.1 Diarthrodial joint structure and vascular supply. Cartilage has no direct blood supply but is nourished by nutrients diffused from synovial membrane through synovial fluid (note that subchondral vasculature also supplies essential nutrients) (www.holistichorsehealth.com).

Cartilage protects the underlying bone by its resistance to various forces, e.g. compressive, tensile and shearing stresses. Articular cartilage has no vascular supply but derives the essential nutrients by diffusion through synovial fluid from the vascular network within synovial membrane (Levick 1995). Articular cartilage can be divided into four zones according to the arrangement of chondrocytes and matrix microstructure as described below (Freeman 1979):-

1. Superficial zone; adjacent to the synovial space. Cells have discoidal shape. Both cells and the matrix fibres are oriented parallel to the surface of cartilage.

2. Intermediate zone; cells are evenly distributed and of spherical shape. Fibres arrange themselves into an interlacing meshwork.
3. Deep zone; spherical cells are arranged in columns. Fibres are mostly in radial orientation to the surface.
4. Calcified zone; adjacent to the subchondral bone. Matrix is deposited and mineralised by crystals of calcium salts. There are only few cells in this zone.

Chondrocytes

Chondrocytes are the only cell type resident in cartilage. Although cartilage contains relatively small number of cells, which comprise approximately 1-10% of the tissue volume (Hammerman and Schubert 1962), they can be very active. Chondrocytes are responsible for cartilage matrix synthesis and resorption to maintain the balance of the tissue turnover. Chondrocytes from immature cartilage are capable of undergoing cell division, in contrast to those from adult tissue, whose mitosis is almost undetectable. Chondrocyte morphology and metabolism can be altered as consequences of cell-cell interaction and between the cells and surrounding environment. For example, chondrocytes maintained in three-dimension (3-D) environment such as in pellet, suspension or alginate bead cultures are spherical in shape and much more active in matrix synthesis than those grown in monolayer, which are elongated in shape and active in DNA synthesis and cell division (Stockwell 1979). These differentiations of

chondrocytes are also interchangeable. Dedifferentiated monolayer chondrocytes, which are fibroblastic, are able to re-differentiate back to be chondrogenic when they are re-cultured in 3-D structures. However, this property is limited only to the early passaged chondrocytes (Domm et al. 2002; Murphy and Polak 2004).

Cartilage extracellular matrix

Cartilage matrix has important roles not only to facilitate joint function but also to maintain homeostasis of the chondrocyte environment. Although cartilage seems to be a very simple tissue and homogeneous in gross appearance, there is histological variation depending on various factors such as species, age, sites and functions. Articular cartilage is categorized as a hyaline cartilage, containing a complex of macromolecular structure including a collagenous fibril meshwork, primarily made of type II collagen, a highly hydrated ground substance composed mainly of proteoglycans (mainly aggrecan), and other non-collagenous protein, including link protein, fibronectin, cartilage oligomeric matrix protein and small proteoglycans (Fig 1.2). Cartilage has a very high water content, which explains the glossy appearance of the tissue, approximately 70% by weight in mature cartilage. On a dry weight basis, equine articular cartilage contains about 50% collagen, 35% proteoglycan, 10% glycoprotein, and other minor components (McIlwraith and Trotter 1996).

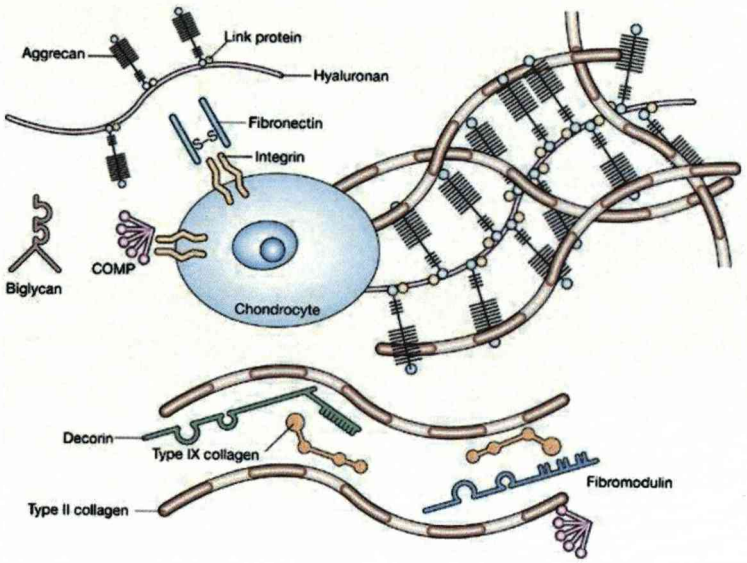

Fig 1.2 The example of major components of hyaline cartilage extracellular matrix (Chen et al. 2006). Collagens (type II, IX and XI), proteoglycans (primarily aggrecan and other small PGs such as decorin, biglycan and fibromodulin), and non-collagenous protein (link protein, fibronectin and cartilage oligomeric matrix protein/ COMP) are shown.

Collagens

The collagens are a family of proteins that are distributed throughout the body providing a broad range of extracellular matrix functions. At least 30 members of the collagen family have been identified and classified (reviewed by Kadler et al. 2007) (Table 1.1). Collagens are synthesized as procollagen molecule containing N- and C-terminal which are cleavable by N- and C-proteinases (Greenspan 2005). Each collagen fibre is made up of tropocollagen molecules, which consist of three polypeptide α-chain forming a triple helix structure. Differences among the various types of collagens are due to varying combinations and modifications of the different chains within the triple helix (homotrimer; same three α-chains, heterotrimer; three different α-chains or heterotypic; combination of two α-

chains from one collagen type and an α-chain from another type (Cremer et al. 1998). The peptide sequence within the collagen triple helix is (Gly-X-Y) where X and Y are often proline and hydroxyproline, respectively. The helical structure provides resistance to proteolytic enzymes except matrix metalloproteinases (Cremer et al. 1998).

Collagens provide structural support, strength and biological properties. There are many types of collagen expressed in articular cartilage (Table 1.2). Among these, type II collagen is the major type of collagen in hyaline cartilage. It copolymerises with two other minor collagens, called types IX

Table 1.1 Collagens and their classification (adapted from Kadler et al. 2007)

Classification	Collagen
Fibril forming	I, II, III, V, XI, XXIV, XXVII,
Network forming	IV, VIII, X
Beaded filament forming	VI, XXVI, XXVIII
FACIT (Fiber-Associated Collagens with Interrupted Triple helices)	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
Transmembrane	XIII, XVII, XXIII, XXV, Ectodysplasin A, Gliomedin
Endostatins	XV, XVIII
Anchoring fibril	VII

and XI to form a complex fibril heteropolymeric or heterotypic fibril (Fig 1.3).The dense fibrillar collagen network is embedded within a very high concentration of proteoglycan aggregated with hyaluronan, which draws water into the tissue and expands the collagen network (McIlwraith and Trotter 1996). Type VI collagen is found in the pericellular region and is

thought to help bind the cell surface to matrix collagen and proteoglycans (Poole et al. 2001). Destruction of the collagen network is believed to be an irreversible stage of cartilage damage as chondrocytes are poor at replacing the collagen architecture and this leads to many joint problems such as osteoarthritis (Eyre 2004).

Table 1.2 Collagen molecules of articular cartilage and their functions (adapted from Poole et al. 2001)

Collagen	Comments	Function
Type II	Principle component of fibril	Tensile strength
Type VI	Forms microfibrils in pericellular sites	Unknown
Type IX	Cross-linked to surface of fibril	Tensile properties and/or fibril-interfibrillar connections
Type X	Associated with fibril and present in pericellular network. Only synthesized by hypertrophic chondrocytes. Only usually present in calcified layer	Unclear but may add structural support
Type XI	Present within and on fibrils	Nucleates fibril formation
Type XII and XIV	Each is homotrimeric	Probably part of fibril

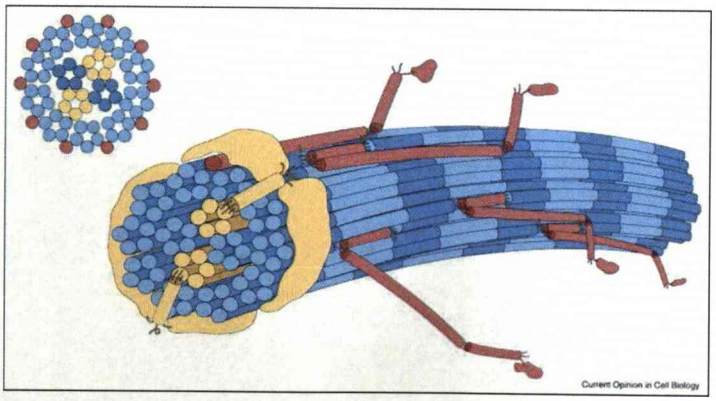

Fig 1.3 Schematic of heteropolymer of collagen II/IX/XI in a thin cartilage collagen fibril. (Blue: collagen II molecule; yellow: collagen XI molecule; red: collagen IX molecule). Collagen XI microfibrils are at the fibril core, surrounded by collagen II microfibril and collagen IX at the surface. The N-terminal of collagen XI are shown extending from the core microfibrils onto the fibril surface (Kadler et al. 2008).

Proteoglycan

Proteoglycan, a class of glycosylated proteins, provides a compressive resistance to cartilage by acting as a sponge that absorbs water into the tissue. Proteoglycan molecules consist of core proteins to which are (covalently) attached chains of glycosaminoglycans (GAGs). GAG chains are polysaccharides made of repeating disaccharides, typically a repeat of 40-100 units. Most GAGs such as chondroitin sulphate, keratan sulphate, dermatan sulphate, heparan sulphate are modified by sulphation within their disaccharide units, with hyaluronan being an exception. The GAG components of proteoglycan are shown in Table 1.3. The biological function providing compressive resistance of proteoglycans, primarily aggrecan and also perhaps versican (Matsumoto et al. 2006), derive mostly from the strong negative charge of glycosminoglycan sulphation that attracts counter ions which causes water influx due to the Gibbs-Donnan equilibrium (Kovach 1995).

Aggrecan is the major proteoglycan of cartilage, found in complex multimolecular aggregates comprised of numerous monomers non-covalently bound to hyaluronic acid (Kiani et al. 2002). The core protein has three globular domains, which are G1, G2 and G3, and three extended domains; IGD, KS and CS (Fig 1.4). Aggrecan forms a stable complex in ECM by interaction between its G1 domain and hyaluronan, which is strengthened by link proteins. Between G1 and G2 domain is an inter-globular-domain (IGD) (Kiani et al. 2002). The chondroitin sulphate

chains, either 4-sulphated, 6-sulphated, or sometimes both, are attached to the long, extended domain between G2 and G3. Keratan sulphate chains are more widely distributed but preferentially located towards the N terminus.

Table 1.3 Glysosaminoglycan components of proteoglycan found in cartilage (adapted from McIlwraith and Trotter 1996)

Glycosaminoglycan name	Primary core protein	Wet weight of cartilage (%)*	Wet weight (nmol/gm)*	Other names	Repeating unit in glycosaminoglycan
Chondroitin sulphate	Aggrecan	5-10	1-10	PG-LA1	N-acetylgalactosamine-β(1-4)-glucuronic acid-β(1-3)
Dermatan sulfate	Decorin	0.03-0.12	0.3-0.6	PG-S2 PGII	N-acetylgalactosamine-β(1-4)-glucuronic acid-β(1-3)
	Biglycan	0.06-0.24	0.25-0.5	PG-S1 PGI	N-acetylgalactosamine-α(1-4)-iduronic acid-α(1-3)
Keratan sulfate	Aggrecan			PG-LA1	N-acetylglucosamine-β(1-3)-galactose-β(1-4)
	Fibromodulin	0.1-0.3	1.5-5	59-kDa protein	N-acetylglucosamine-β(1-3)-galactose-β(1-4)
Hyaluronan	None	0.05-0.25	0.03-0.08	Hyaluronic acid	N-acetylglucosamine-β(1-4)-glucuronic acid-β(1-3)

* Data are approximate; these molecules are particularly heterogenous, and estimates of their molecular mass vary by as much as 50%. In addition, calculation of the percentage wet weight of cartilage are prone to error based on the efficiency of extraction. To further complicate matters, the amounts of most components vary with age and exact tissue source. These figures are thus only a rough guide.

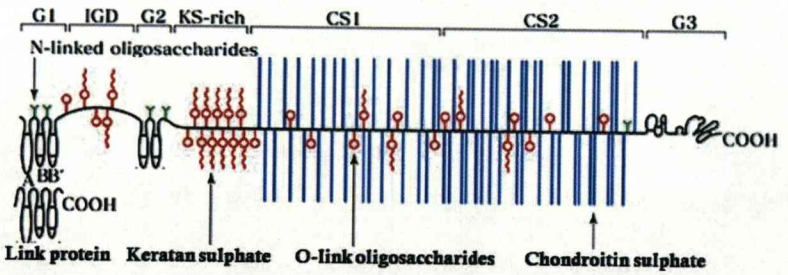

Fig 1.4 Schematic representation of aggrecan monomer and link protein structure (adapted from Dudhia et al. 1990). The domains [G1, G2, G3 and interglobular-domain (IGD)] with keratan sulphate- and chondroitin sulphate- rich regions are shown.

Cartilage oligomeric matrix protein (COMP)

Cartilage oligomeric matrix protein, also called thrombospondin 5, is an abundant non-collagenous protein component of cartilage matrix (Hedbom et al. 1992). COMP has a pentameric form of five subunits arranged in a bouquet-like structure, a central cylindrical structure radial with five globular-domain ended arms (Morgelin et al. 1992) (Fig 1.5). COMP can be degraded by ADAMTS-12 (Liu et al. 2006) and has been used as a biomarker of cartilage destruction in osteoarthritis and rheumatoid arthritis (Dahlberg et al. 1994; Lohmander et al. 1994; Mansson et al. 1995; Sharif et al. 1995) although COMP is also a marker of synovitis as synovial cells can also produce this molecule (Recklies et al. 1998). Mutation of the COMP gene results in skeletal disorders such as pseudoachondroplasia and multiple epiphyseal dysplasia (Briggs et al. 1995; Hecht et al. 1995). However, the precise function of COMP in cartilage is still not clear. COMP is distributed throughout cartilage matrix but preferentially localised in the interterritorial zone (Murphy et al. 1999). It has been shown that COMP can interact with type II and IX collagens (Rosenberg et al. 1998; Holden et al. 2001; Thur et al. 2001) and also aggrecan (Chen et al. 2007) (Fig 1.5). It is therefore suggested that COMP may play a role in cartilage matrix integrity. Recently, COMP was shown to have anti-apoptotic properties for chondrocytes (Gagarina et al. 2008) and can support chondrocyte attachment to the ECM through integrins (Chen et al.

2005). Taken together, COMP has complex roles in maintaining both cartilage matrix and chondrocytes.

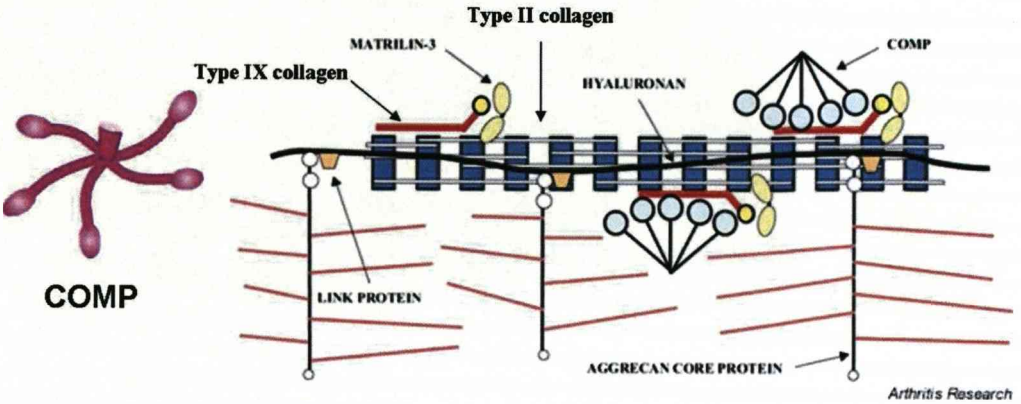

Fig 1.5 Diagram of cartilage oligomeric matrix protein (COMP) structure and interaction with other cartilage matrix molecules e.g. collagen fibrils and aggrecan (adapted from http://www.cmb.lu.se/ctb/html/COMP.htm and Reginato and Olsen 2002).

Cartilage homeostasis

Cartilage homeostasis reflects a balance between synthesis and degradation of matrix components, which is in part mediated by chondrocytes. Surrounding tissues such as synovial membrane and subchondral bone are also influential in signalling matrix turnover processes especially under pathological conditions. Cartilage is a dynamic tissue, in which there is remodelling of the matrix and degradation and synthesis of matrix molecules. However, in the adult, turnover of collagen for example can be really slow with t1/2 values in the order of 70+ years (Maroudas et al. 1992). Enzyme inhibitors and anti-inflammatory cytokines participate in the anabolic process to stimulate extracellular matrix formation and cell proliferation. On the other hand, proinflammatory cytokines and enzymes

participate in the catabolic process, which results in the destruction of the cartilage matrix and a reduction of cell proliferation. The enzymes that play a key role in the degradation of the cartilage matrix are the proteases, which are inducible by a number of cytokines and growth factors. Among the different families of proteases, matrix metalloproteinases (MMPs) are the most commonly associated with ECM degradation process. The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) enzymes are also involved in cleaving hyaluronan-binding proteoglycan (hyalectan). Under physiologic conditions, the net activities of these enzymes are regulated by their physiological inhibitors called the tissue inhibitor of metalloproteinases (TIMPs) (Murphy et al. 1994).

Cytokines

Cytokines are soluble or cell-surface molecules that play an essential role in mediating cell-cell interactions. In general, cytokines act on cells that produced them or in the area surrounding the cells, although in certain situations they may enter the circulation and act in an endocrine fashion. They interact with target cells by binding to specific receptors present on the cell surface. It is possible to classify (Table 1.4) the cytokines into 4 categories (Goldring and Goldring 2004):-

1. Catabolic cytokines; regulate cartilage remodeling, acting on target cells to increase synthesis and release of products that enhance matrix degradation.

2. Anticatabolic cytokines; tending to inhibit or antagonise the activity of the catabolic cytokines.

3. Anabolic cytokines (e.g. growth factors); acting on chondrocytes to increase synthetic activity

4. Modulatory cytokines; these cytokines are grouped based on their capacity to modulate the activities of the other cytokines

Table 1.4 Chondrocyte-Cytokine Interactions (Goldring and Goldring 2004)

Catabolic	Modulatory	Anti-Catabolic	Anabolic
IL-1	IL-6	IL-4	IGF-1
TNF-α	LIF	IL-10	TGF-β1,2,3
IL-17	IL-11	IL-13	BMP-2,4,6,7,9,13
IL-18		IL-1ra	
OSM			

1. Interleukin 1 (IL-1)

IL-1 is generally found to induce a catabolic response. It induces chondrocyte-mediated cartilage degradation by stimulating the degradative proteinases involved in this process, inhibiting the synthesis of macromolecules essential for matrix integrity, and also inhibiting proliferation of chondrocytes induced by serum or TGF (Goldring and Goldring 2004). There are three members of the IL-1 family, IL-1 alpha, IL-1 beta and IL-1Ra. The first two isoforms bind to the same cellular receptor that transmits intracellular signals in inflammation whilst IL-1Ra

competes for their receptor binding, blocking their role in immune activation (Dinarello 1994).

2. Tumor Necrosis Factor-alpha (TNF-α)

TNF is a member of a superfamily of proteins, each with 157 amino acids, which induce necrosis of tumor cells and posses a wide range of proinflammatory actions. TNF established its role in tissue destruction in rheumatoid arthritis and related inflammatory joint disorders (van den Berg 2001; Van den Berg 2002). It has similar effects to IL-1, stimulating matrix-degrading protease activity levels and suppressing cartilage matrix synthesis, effect that are susceptible to synergistic enhancement when these cytokines are combined together (Berenbaum et al. 1996).

3. Oncostatin M (OSM)

OSM is a member of the IL-6 family of cytokines (Rose and Bruce 1991). It is considered a multifunctional cytokine that affects the growth and differentiation of several cell types and participates in both catabolic and anabolic processes. Whilst increasing mediators that inhibit inflammatory mechanism such as IL-1Ra and TIMPs, OSM also stimulates the inflammatory response. However, the influence of OSM in matrix degradation is obviously magnified when it is combined with IL-1. There are several studies using human and bovine cartilage, showing a synergistic effect between OSM and IL-1 in upregulation of MMP genes (Hui et al. 2001; Barksby et al. 2006 a) and increasing cartilage matrix degradation (Milner et al. 2001; Morgan et al. 2006).

4. Transforming Growth Factor-beta (TGF-β)

The transforming growth factor beta family of molecules is a group of anabolic cytokines that are involved in several extracellular matrix formation processes such as wound healing, skeletal morphogenesis and carcinogenesis (Chin et al. 2004). TGF-β also promotes cartilage formation (Frenkel et al. 2000). Specifically, TGF-β has been reported as being able to promote type II and type IX collagen synthesis and also aggrecan synthesis (Chu et al. 1995) and is also able to down-regulate the effects of cytokines that stimulate chondrocyte catabolic responses (van Beuningen et al. 1993).

5. Insulin-like Growth Factor-1 (IGF-1)

IGF-1 is a peptide with a potent anabolic impact on cartilage homeostasis. It stimulates the proliferation of chondrocytes and favours matrix synthesis. It has been shown to drive equine cartilage proteoglycan and collagen matrix production *in vitro* (Fortier et al. 1999). IGF-1 also blocks collagen breakdown and down regulates MMP-1, 3, 8 and 13 mRNA expression in bovine nasal cartilage (Hui et al. 2001).

Matrix metalloproteinases (MMPs)

The matrix metalloproteinases (MMPs) are a superfamily of proteolytic enzymes responsible for extracellular matrix degradation (Birkedal-Hansen et al. 1993). MMPs are necessary in tissue remodelling and are involved in wound healing, angiogenesis, and tumour cell metastasis. They are called metalloproteinases because they need zinc or calcium atoms for their

function. MMPs are secreted as a latent form, which is determined by their N-terminal propeptide of 77-87 amino acids with a conserved region. The interaction between cysteine within the conserved region and zinc is the characteristic of the proenzyme form. Destablisation of this interaction by sequential cleavages can modify the enzyme conformation and allows activation to the active form (Murphy et al. 1994). There are currently 24 members (Nagase et al. 2006) within this superfamily, which can be subdivided into six major categories, collagenases; gelatinases; stromelysins; matrilysins, membrane-type (MT) MMP and others, according to their domain organisation and substrate preference (Nagase et al. 2006). MMP-1, -8, -13 and -18 are grouped as collagenases, which cleave collagen types I and II into ¾ and ¼ fragments. Among these, collagenase 3 (MMP-13) is a powerful collagenolytic enzyme that preferentially cleaves type II collagen, the main collagen in cartilage. Gelatinases (MMP-2 and MMP-9) have a specific domain, called fibronectin type II motif (Fig 1.6), which binds to gelatin and also collagen, allowing the enzyme to digest these substrates. However, the collagenolytic activity of gelatinases is much weaker than MMPs from the collagenases group (Nagase et al. 2006). Stromelysins are involved in many MMP activations, degradation of proteoglycans and depolymerisation of the heterotypic collagen fibril by cleaning the non-collagenous regions of collagen type II and XI (Wu and Eyre 1995). Although their domain arrangement is similar to collagenases, stromelysins

have no collagenolytic activity at all (Nagase et al. 2006). Membrane-Type matrix metalloproteinases (MT-MMPs) are activated intracellularly before they are transferred to the cell surface so that these enzymes can work in this precise location. MT-MMPs also participate in proMMP-2 and proMMP-13 activation (Knäuper et al. 1996 b).

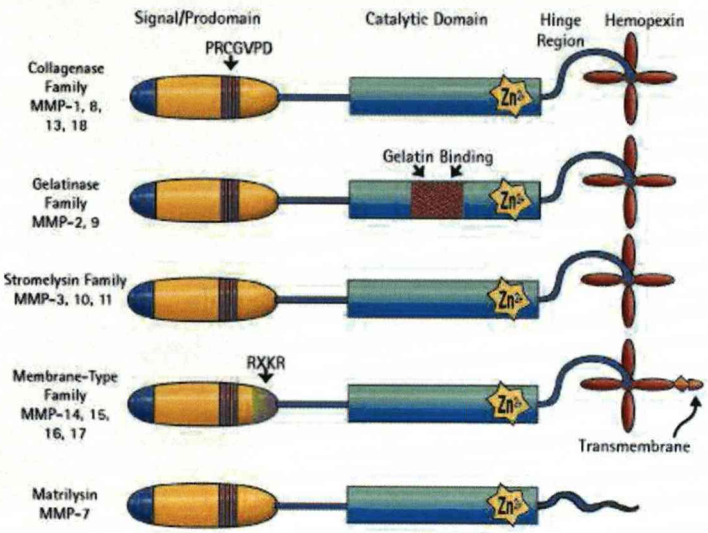

Fig 1.6 Structural domains of matrix metalloproteinases. The schematic shows signal peptides, prodomains, catalytic domains, hinge regions and hemopexin-like domains of each MMP type. The transmembrane domain is shown in MT-MMP, while hemopexin-like domain is not presented in matrilysin. (http://www.emdbiosciences.com/html/cbc/matrix_metalloproteinase_MMP.htm)

MMPs can be regulated at many levels. Their expression is transcriptionally controlled by inflammatory cytokines, growth factors, hormones, cell to cell and cell to matrix interactions (Nagase and Woessner 1999). MMP activity is mediated by activation of the zymogens, which can be inhibited by the endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)

The ADAMTS are a group of proteinases which are also found in cartilage. Nineteen human ADAMTS have been identified. ADAMTS-1, -4, -5, -8, -9 and -15 cleave the hyalectan (hyaluronan-binding proteoglycan) (Jones and Riley 2005) and ADAMTS-4 and -5 have been implicated as the aggrecanases involved in aggrecan degradation in osteoarthritis (OA) (Malfait et al. 2002). Studies in ADAMTS-4 and -5 knockout mice revealed that they were phenotypically normal and indistinguishable from wild-type littermates, indicating that each enzyme was dispensable for normal development (Glasson et al. 2004; Glasson et al. 2005; Stanton et al. 2005).

Tissue inhibitors of metalloproteinases (TIMPs).

The activity of the metalloproteinases is regulated by the enzymes' physiological inhibitors and activators. The endogenous inhibitors of the MMPs are the TIMPs (tissue inhibitors of metalloproteinases). Four TIMPs (TIMPs1-4) have been identified to date (Gomez et al. 1997). TIMPs consist of 184-194 amino acids, which are subdivided into an N-terminal and a C-terminal subdomains. (Nagase et al. 2006). TIMPs form a 1:1 complex structure with the catalytic domain of MMPs to effect inhibition and all TIMPs can inhibit every identified MMPs so far but to a different degree (Nagase et al. 2006). However, TIMP-3 has been implicated as a

major regulator of metalloproteinase activities *in vivo* (Fata et al. 2001; Leco et al. 2001).

Metabolic energy of chondrocytes

The primary energy resource of chondrocyte metabolism is mainly provided by extracellular glucose, which is metabolised as a substrate for ATP production to control cellular respiration and homeostasis (Richardson et al. 2003; Windhaber et al. 2003). In addition to the energy supply, glucose is the carbon skeleton resource of many biological substances, such as protein, lipid, nucleic acids and complex polysaccharides, and is also an important component for extracellular matrix synthesis e.g. it is incorporated alongside glusosamine sulphate and other sulphated sugars to produce glycosaminoglycans (Windhaber et al. 2003).

Since the cell membrane is impermeable to glucose, transport of glucose into and out of the cell is manipulated by facilitated diffusion through membrane protein carriers called glucose transporters or GLUTs. GLUTs are located in the plasma membrane and have glucose binding sites both inside and outside the membrane. Besides transporting glucose, GLUTs also play another role as transporters of diascorbic acid, which is an important co-factor of collagen and glucosamine synthesis (Shikhman et al. 2004).

Glucose transporters are part of the GLUT or SLC2A gene family which belong to a larger superfamily of proteins known as the major facilitator superfamily (MFS) (Saier et al. 1999). GLUTs are distinct from the SGLT/SLC5A family of Na^+-dependent sugar transporters (Wood and Trayhurn 2003). GLUT proteins are characterised by the presence of 12 membrane spanning helices and several conserved sequence motifs (Pessin and Bell 1992) (Fig 1.7). GLUT isoforms (at least 13 members have been identified) differ in their substrate specificity and tissue distribution (Table 1.5). Human chondrocytes express a number of glucose transporter proteins (Table 1.6).

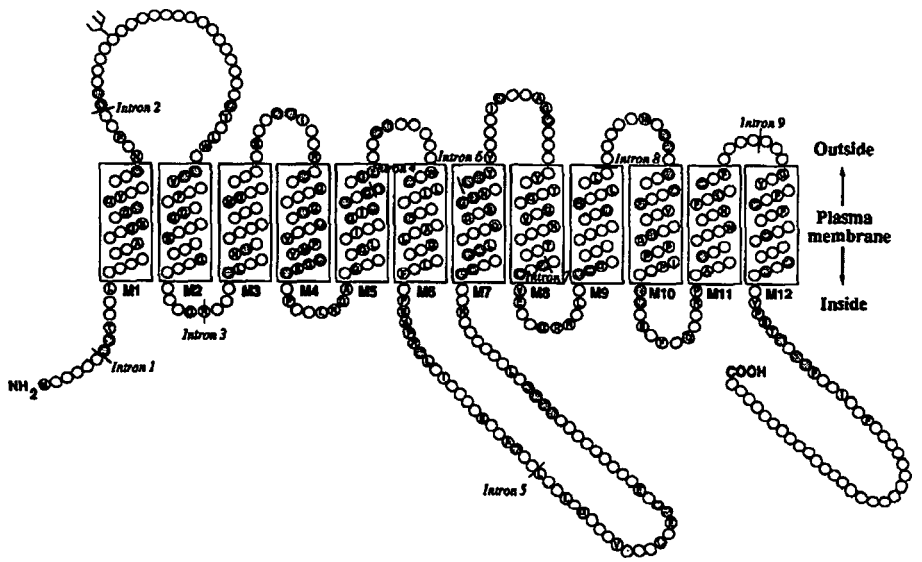

Fig 1.7 Glucose transporter structure (Pessin and Bell 1992) showing 12 membran-spanning segments (M1-M12) and the position of exon boundaries in the protein.

Table 1.5 GLUT expression in mammalian tissues (adapted from Mobasheri et al. 2002 a)

GLUTs	Main tissues expressing GLUTs
GLUT1	Red blood cell and endothelial cells
GLUT2	Liver and pancreatic cells
GLUT3	Neuron and platelet
GLUT4	Muscle and adipose tissue
GLUT5	Intestine and testis
GLUT6	See GLUT9
GLUT7	Liver and other glyconeogenic tissues
GLUT8	Testis and placenta
GLUT9	Kidney and liver (also in placenta, lung, blood and peripheral leukocytes, heart, skeletal mucle, spleen and brain)
GLUT10	Liver and pancreas
GLUT11	Heart and skeletal muscle
GLUT12	Skeletal muscle, adipose tissue and small intestine
GLUT13	H^+myo-inositol transporter and brain

Table 1.6 GLUTs in human cartilage (adapted from Richardson et al. 2003)

Protein	Gene name	Proposed physiological Functions
GLUT1	SLC2A1	Housekeeping glucose transporter; ubiquitously expressed. Its expression appears to be up-regulated by glucose deprivation and prolonged hypoxia ischemia.
GLUT3	SLC2A3	Fast glucose transporter. High affinity transporter specialized for glucose uptake where substrate concentration are low.
GLUT4	SLC2A4	Insulin sensitive glucose transporter (expressed only in developing cartilage)
GLUT5	SLC2A5	Fructose transporter
GLUT6 (alias GLUT9)	SLC2A6	See GLUT 9
GLUT8 (alias GLUT X1)	SLC2A8	Down regulated by glucose deprivation and prolong hypoxia
GLUT9 (alias GLUT X)	SLC2A9	Glucose transporter expressed in kidney, liver, and cartilage
GLUT11	SLC2A11	Glucose transporter with several transcription variants (product of mRNA splicing)
GLUT12	SLC2A12	GLUT-12 may comprise a second insulin-sensitive glucose transport system

The avascular nature of cartilage results in low supplies of glucose and oxygen to chondrocytes. Limited oxygen drives chondrocytes to generate energy mainly by anaerobic glycolysis (Lee and Urban 1997), which produces 18-19 times less ATP per molecule of glucose than aerobic respiration (Ahlqvist 1984) (Fig 1.8). Therefore, chondrocytes must be able to manage glucose transport and metabolism effectively to provide sufficient energy within the anatomical and physiological constraints of cartilage.

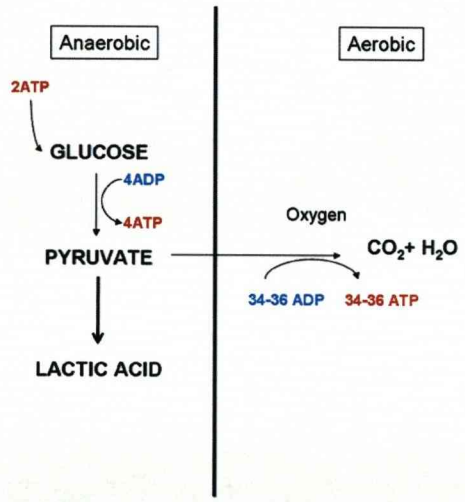

Fig 1.8 Cellular energy generation from anaerobic and aerobic respiration. Under aerobic conditions, a cell gains net 36-38 ATPs from glycolysis and oxidative phosphorylation per molecule of glucose while under anaerobic condition, cell produces 2 ATPs from glycolysis only.

Among GLUTs expressed within cartilage, GLUT1 and GLUT3 are hypoxia responsive isoforms and are regulated by the transcription factor and oxygen sensor protein, hypoxia-inducible factor 1 (HIF-1) (Mobasheri et al. 2005 a).

Cartilage physiology under hypoxia

Articular cartilage is an avascular tissue. Nutrients and oxygen are delivered by diffusion from blood vessels of the underlying bone and from synovial fluid, which lubricates the joint (Lee and Urban 1997). Because of this limitation, compared with other tissues, which are maintained by capillary networks, cartilage is under hypoxic and nutrient deficient conditions. It is well known that there is a physiological oxygenation gradient within articular cartilage. It has been estimated that chondrocytes at the articular surface are exposed to 6-10% oxygen while the deepest layer can access only 1% or less oxygen (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971; Silver 1975; Kiaer et al. 1988; Ferrell and Najafipour 1992). However, chondrocytes appear to be very well adapted to such environments (Rajpurohit et al. 1996). It was found that the anabolism and cell survival of cartilage were maximized when it was cultured *in vitro* under low oxygen tension compared to normoxic and anoxic conditions (Grimshaw and Mason 2000; Hansen et al. 2001; Domm et al. 2002; Schneider et al. 2004). Therefore it may be that hypoxia is the natural or physiological state of chondrocytes.

Avascular tissue such as cartilage is able to modulate its physiology to survive in low oxygen environment by organisation of hypoxia-inducible factor-1 (HIF-1), which is responsive to cellular oxygen concentration. The important roles of HIF-1 in cell survival have been reported (Schipani et al.

2001; Mazure et al. 2004; Yudoh et al. 2005). It is known that HIF-1 rescues oxygen-scarce cells by regulation of vital gene expression.

HIF-1 and its regulators

HIF-1 is a heterodimer composed of 2 subunits, HIF-1α and HIF-1β. HIF-1β is expressed constitutively while HIF-1α is hypoxic responsive (Semenza 2001 a; Semenza 2001 b). Both of them are relatively large proteins in size and contain a basic helix-loop-helix motif (bHLH) (Déry et al. 2005). The basic domain is essential for DNA binding while the HLH domain is responsible for subunit dimerization. Another common structure of both proteins is the Per-ARNT-Sim (PAS) domain, which identifies a protein superfamily (Fig 1.9). The instability of HIF-1α under high oxygen level comes from its unique structure, the oxygen-dependent degradation (ODD) domain, found between residues 401-603. This domain is highly oxygen-regulated.

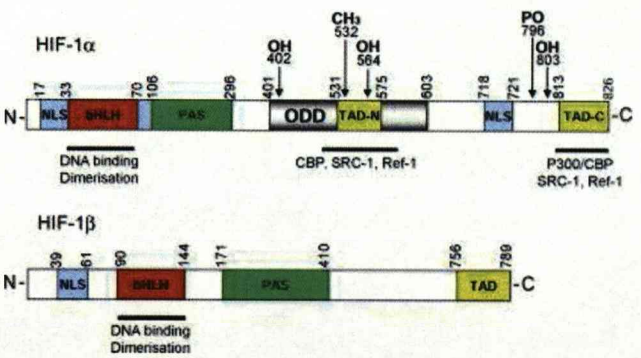

Fig 1.9 HIF-1α and HIF-1β structures (Déry et al. 2005) . Functional domains and binding domains with co-factors are shown. Hydroxylation, acetylation and phosphorylation sites are indicated. bHLH = basic helix-loop-helix; ODD = oxygen-dependent degradation domain; TAD-N and TAD-C = N- and C-terminal transactivatioin domain; NLS = nuclear localisation signal; PAS = Per-ARNT-Sim.

The main regulation of HIF-1 by oxygen is performed on the α subunit. HIF-1α has been found to be extremely labile under normoxic condition with a half life of less than 5 minutes (Déry et al. 2005). This is due to destruction of the α subunit which results in lack of dimerisation to another subunit and blocks the functional HIF-1 complex formation. The stability of HIF-1α is regulated by various factors, including HIF-prolyl 4-hydroxylases (PHDs)(Mazure et al. 2004). PHDs are iron-dependent enzymes, which comprise 3 homologues; PHD1, PHD2 and PHD3 (Masson et al. 2001). These enzymes have been shown to be able to hydroxylate the proline residues, Pro^{402} and Pro^{564}, which are located in the ODD domain of human HIF-1α. This process needs oxygen to transform prolines into hydroxyprolines, which can be recognized by von Hippel-Lindau tumour suppressor protein (pVHL) and become the destroyed target of the proteosome pathway. von Hippel-Lindau tumor suppressor protein (pVHL) has been shown to be the recognition component of the E3 ubiquitin-protein ligase complex (Lisztwan et al. 1999). The capture of this protein and hydroxylated HIF-1α, therefore, activate the destruction of HIF-1α by ubiquitin-dependent proteolysis.

Under normoxic condition, HIF-1α is mainly destroyed by hydroxylation at two proline residues, Pro^{402} and Pro^{564} into hydroxyprolines. It has been shown that PHD2 plays the major role in this process (Berra et al. 2003).

However, under long-term hypoxic stress, PHD1 and PHD3 have been reported to be involved (Berra et al. 2003). On the other hand, under hypoxic conditions, or via hypoxic mimicking effects of iron antagonists such as cobalt chloride, the hydroxylation of HIF-1α by PHD is blocked (Déry et al. 2005). HIF-1α is released from pVHL and translocates into the nucleus, where it heterodimerizes with HIF-1β to form the HIF-1 complex. The kinetics of HIF-1α in various tissues under normoxia and hypoxia has been reported (Stroka et al. 2001). This complex molecule binds to the HIF-1 binding site (HBS) of hypoxic response elements (HRE) in the target gene and starts to regulate the transcription of its downstream genes (Fig 1.10) (Semenza 2001 a).

As seen in other cells, HIF-1α has been shown to be responsible for energy generation and cellular survival of hypoxic chondrocytes. HIF-1α null chondrocytes cannot maintain their viability, energy generation, and matrix production under both normoxic and hypoxic conditions (Yudoh et al. 2005). Also, HIF-1α null chondrocytes showed accelerated IL-1β-induced apoptosis, suggesting that HIF-1α has an important role in the survival of chondrocytes (Yudoh et al. 2005).

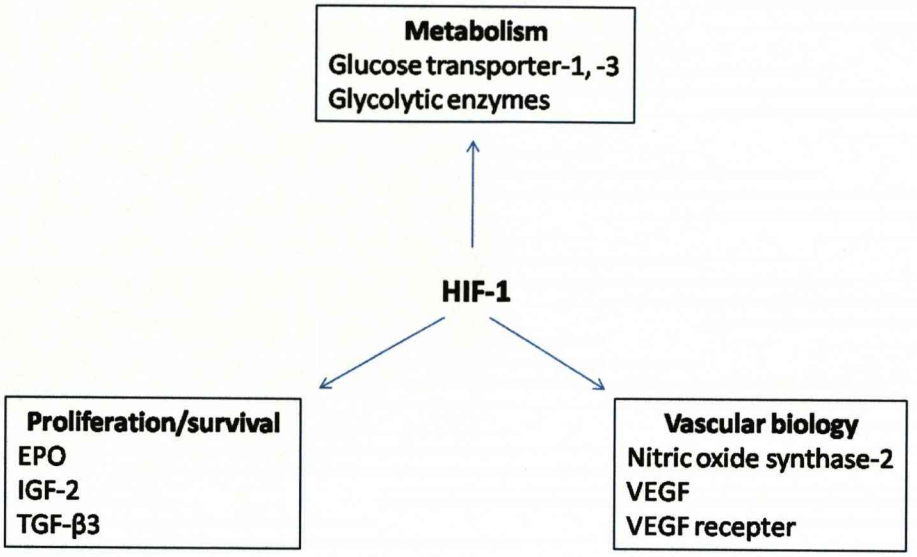

Fig 1.10 Examples of HIF-1 target genes (adapted from Semenza 2001 a). EPO = erythropoietin; IGF = insulin-like growth factor; TGF-β = transforming growth factor-beta; VEGF = vascular endothelial growth factor.

Hypoxia and Joint disease

Joint disease is one of the most important causes of lameness in horse and has a major impact on equine athletic performance. In Great Britain, a study in 1985 found that the greatest cause of attrition of young Thoroughbreds from racing was lameness and among these, joint diseases figured prominently (Rossdale et al. 1985). Traumatic joint injury represents one of the most common causes of joint diseases of the horse and results in osteoarthritis in most severe injuries or following inadequate treatment. Osteoarthritis (OA; also called degenerative joint disease) is the stage of progressive and permanent loss of articular cartilage. It involves all joint structures undergoing morphological alterations while it is progressing. Another common joint abnormality in the horse is

osteochondritis dissecans (OCD), which is categorised as a developmental orthopaedic disease. It involves a failure of endochondral ossification which is associated with a failure of vascular invasion from the underlying subchondral bone, leading to development of dissecting lesion of cartilage from the underlying subchondral bone. The abnormal cartilage in horses can become detached and they then undergo endochondral ossification at a later date (McIlwraith 2002).

Changes in the synovial membrane due to inflammatory processes at the beginning of OA may seriously alter the oxygen transfer from the capillaries of the synovium to the synovial fluid, which is subject to oxygen fluctuation as a consequence of ischemia-reperfusion, pathologic acceleration of tissue metabolism and sustained abnormal strains on the joint (Blake et al. 1989). Similarly to pathologic articular changes in OCD, lack of blood supply from underlying bone can cause ischemia within the cartilage. It has been reported that a considerably reduced oxygen transfer across the synovial membrane occurs in the acute stage of experimental osteoarthritis (Svalastoga and Grønlund 1985). Another study using *in vivo* measurement has provided evidence that oxygen tensions are decreased in the osteoarthritic joint (Kiaer et al. 1988). Two separate studies in rabbit and horse also showed significant increases in oxygen consumption in acutely osteoarthritic and inflamed joints indicating an increase in oxygen

demand in pathologic conditions (Svalastoga and Grønlund 1985; Hardy et al. 1998).

The survival and function of chondrocytes depends on their adaptation to surrounding conditions. Degeneration of articular cartilage may directly influence the chondrocyte environment (Yudoh et al. 2005), especially cellular adaptation to changes in oxygen level, and could directly affect the maintenance of articular cartilage matrix. It has been demonstrated that HIF-1α is expressed in OA cartilage (Coimbra et al. 2004) and this was suggested to be a chondrocyte survival factor in this disease (Yudoh et al. 2005). Failure in adaptation to oxygen alteration by chondrocytes may cause a serious metabolic imbalance and lead to deterioration of the cartilage matrix.

Hypoxia and chondrogenicity

Articular cartilage lacks the ability to heal itself due to the avascular nature of this tissue. Therefore, numerous studies have explored therapies for repairing damaged cartilage such as tissue engineering. However, it remains a major challenge to create a non-vascularised homogeneous cartilaginous tissue in this way. Living in a physiologically-limited oxygen environment, chondrocytes may suffer from hyperoxic shock, i.e. damage from reactive oxygen species (Henrotin et al. 2005), when they are cultured in atmospheric conditions. Factors mimicking the *in vivo* environment of articular chondrocytes such as hypoxia might influence their ability to

maintain chondrogenic phenotypes. Changes in behaviour of isolated chondrocytes at different oxygen tension have been reported. A positive effect of hypoxia has been found in equine chondrocyte viability studies (Schneider et al. 2004), on bovine articular cartilage matrix mRNA expression (Barry and Murphy 2004) and production (Domm et al. 2002) and redifferentiation ability in bovine articular cartilage (Domm et al. 2002) and in human nasal cartilage (Malda et al. 2004 a). However, another study in bovine articular cartilage could not identify effect of altered oxygen tension in stabilizing chondrogenicity (Saini and Wick 2004). This controversy results in unclear understanding of the effects of oxygen on cartilage matrix synthesis. Moreover, there is no report, to my knowledge, concerning hypoxia and its influence in neo-cartilage synthesis of equine chondrocytes, which could be useful information for further studies of equine cartilage tissue engineering.

Hypotheses

Low oxygen tension (defined here as equal to or less than 5% O_2) is considered to be physiological condition of articular cartilage. Chondrocyte metabolism observed in culture under higher oxygen tension (approximately 20%) may not reflect cell behaviour in the native tissue. Studies of the effects of low oxygen levels on major chondrocyte functions may provide information applicable to *in vitro* culture systems. There is evidence to support the adaptation of chondrocytes to various oxygen

tensions and several studies have reported beneficial effects of low oxygen level on chondrocyte survival and chondrogenicity. The hypotheses investigated within this thesis are that low oxygen tensions 1) would alter chondrocyte glucose consumption to maintain cellular energy status 2) would antagonise cartilage matrix catabolic processes and 3) favour extracellular matrix synthesis by chondrocytes.

Aims of the study

To investigate the above three hypotheses, the studies are divided into three main parts in order to cover the major functions of equine chondrocytes in cartilage homeostasis using established *in vitro* techniques. The first aim was to investigate changes in basic glucose transportation using monolayer equine chondrocytes under hypoxic environments (also an observation of glucose transporter expression in pathologic cartilage is included). The second aim was to study the effect of hypoxia on cartilage catabolism using cytokine-induced equine cartilage explant model. The final aim was to investigate the effect of hypoxia on cartilage matrix production using the chondrocyte pellet culture model. Also, a preliminary study of hypoxia inducible factor and its regulator using equine chondrocytes is reported in this study.

Chapter II

GENERAL MATERIALS AND METHODS

General reagents

General chemical reagents were from Sigma-Aldrich Company Ltd. and VWR International Ltd. General tissue culture reagents were from Invitrogen Ltd.

Cases and controls

All cartilage samples were provided by Prof. Pete Clegg. Samples were collected in accordance with institutional guidelines with ethical review and written informed consent from the animal owners. Cartilage samples from normal equine joint were used in all studies except study of GLUT1 mRNA expression in osteoarthritis and osteochondritis dissecans cartilage.

Cartilage samples

1. Normal cartilage

Normal equine articular cartilage was obtained post mortem from two sources, a horse abattoir and the Philip Leverhulme Equine Hospital, University of Liverpool. The cartilage from Equine Hospital cases was biopsied from trochlear ridges and medial femoral condyles of the femur of healthy stifle joints of horses aged between 2-25 years old which had been euthanased for clinical reasons not related to joint problems. The cartilage

samples from the abattoir were collected post-mortem from the metacarpophalangeal/tarsophalangeal joints within an hour of euthanasia.

2. Osteochondritis dissecans (OCD) cartilage

Full thickness cartilage biopsies from OCD lesions of the lateral trochlear ridge of the femur (LTRF) were harvested from 5 horses (aged 4-7 years) during therapeutic arthroscopic debridement of the lesions.

3. Osteoarthritic (OA) cartilage

Full thickness cartilage biopsies were harvested immediately post-mortem from the medial femoral condyle of 8 horses (aged 2-9 years) that were euthanased as a consequence of lameness due to severe osteoarthritis of the medial femorotibial joint. Gross post-mortem indicated the presence of osteoarthritis in all cases.

Processing cartilage

Cartilage was transported to the laboratory in Dulbecco's modified Eagles medium (DMEM) with 300unit/ml penicillin G sodium, 300μg/ml streptomycin and 7.5μg/ml amphotericin B. Before any processing, cartilage was rinsed at least three times with the same media. Where tissue was harvested for mRNA isolation, the cartilage was placed in RNAlater™ and then transferred on ice to the laboratory for further processing.

Chondrocyte culture

1. Monolayer cultures

Rinsed cartilage was cut into small pieces and digested overnight with 0.1% type I collagenase (Sigma C-0130; EC 232.582.9 from *Clostridium histolyticum*) in the same rinsing medium at 37°C. The digest was filtered through a 40μm cell strainer and centrifuged at 1400 rpm for 10 minutes. The resulting chondrocytes were washed again by resuspension and centrifugation before culturing as a monolayer in DMEM with 1 g/l glucose, L-Glutamine and pyruvate, supplemented with 10% foetal calf serum, 100 unit/ml penicillin G sodium, 100μg/ml streptomycin and 2.5μg/ml amphotericin B. All subsequent experiments were performed on first passage cells unless stated otherwise, in which case they were 2nd or 3rd passage. Chondrocytes were not used in any experiment beyond the third passage because it has been clearly shown that their phenotype changes after multiple passages (Domm et al. 2002; Murphy and Polak 2004).

2. Encapsulated alginate bead cultures

Low viscosity alginate (Sigma; A-2158) was slowly dissolved in 0.15M NaCl on a warm plate to 1.2% (w/v) and clarified by centrifugation at 3000g for 15 minutes. The solution was autoclaved-sterilised before use. Prepared chondrocytes were washed in 0.15M NaCl prior to being suspended at 30,000 cells per ml of the alginate solution. This cell suspension was gently mixed by pipetting and transfer to a sterile

disposable plastic 5 ml syringe equipped with a 21 gauge needle. The suspended chondrocytes were slowly expressed in a dropwise fashion into 40ml of gently agitated 0.1M $CaCl_2$. The immediately formed alginate gel beads were allowed to set at room temperature for 10 minutes on a roller. The $CaCl_2$ solution was decanted and the beads were washed three times in 0.15M NaCl and once in DMEM. The encapsulated chondrocytes were cultured in a square petri dish with 25ml of DMEM supplemented with 10% FCS and antibiotics, as described in monolayer culture medium except amphotericin B was omitted because of known interference to HIF-1α expression (Pfander et al. 2005). Alginate beads containing cells were allowed to equilibrate for at least one week but not more than six weeks before starting any experiments. To recover the encapsulated chondrocytes, the culture medium was decanted from the alginate beads and the beads were washed twice with 0.15M NaCl. Three volumes of a dissolving buffer containing 0.1M sodium citrate were added per volume of packed beads. The suspension was gently agitated until the beads were completely dissolved. The liberated chondrocytes were pelleted by centrifugation at 1400 rpm for 10 minutes and washed with 0.15M NaCl.

3. Explant cultures

After rinsing, cartilage was cut into small pieces, approximately 2x5x2 mm and cultured in 12 well tissue culture plates: each well contained a piece of cartilage explant with 0.5ml serum-free DMEM containing 25mM HEPES,

100unit/ml penicillin G sodium, 100μg/ml streptomycin and 2.5μg/ml amphotericin B.

4. Pellet culture

The cell pellet culture method was developed in a 96-well plate system by Penick and co-workers (2005) to reduce cost and time to produce chondrogenic aggregates which were in the same quality with those cultured in original 15ml polypropylene tubes (Penick et al. 2005). For freshly isolated chondrocyte pellet cultures, rinsed cartilage was diced into small pieces and chondrocytes were isolated using 0.4% pronase (Sigma P-5147; EC 232.909.5 from *Streptomyces griseus*) at 37°C for 30 minutes followed by 0.3% type II collagenase (Invitrogen 17101-015 from *Clostridium histolyticum*) at 37°C for up to 2 hours with cell harvesting and washing at 30 minute intervals prior to pelleting. For first passage chondrocyte pellet cultures, chondrocytes were isolated and cultured in monolayers as described previously (see monolayer cultures) until the first passage and were trypsinised from tissue culture flasks. Chondrocytes, either fresh or dedifferentiated, were rinsed with phosphate buffer saline and centrifuged for 10 minutes at 1400 rpm. The supernatant was discarded and the cells were resuspended with chondrogenic medium (DMEM F-12 supplemented with 1% insulin-transferin-selenium-x (ITS; Invitrogen), 50μg/ml ascorbic acid, 100units/ml penicillin G sodium, 100μg/ml streptomycin and 2.5μg/ml amphotericin B). The cells were counted in a hemocytometer and the suspension volume was adjusted to give a final cell

density of 1.25 million cells per ml in the chondrogenic medium (to obtain 2.5 x 10^5 cells per pellet). In growth factor treated samples, TGF-β3 was added to cell suspensions to a final concentration of 10ng/ml. Aliquots of 200µl were dispensed into the wells of autoclave-sterilised 96-well, V Bottom, 300 ul PCR plates (Thermo-fast ® 96; AB 0900, ABgene) using large orifice tips to allow smooth delivery. A sterile tissue culture plate lid was placed on the plate and temporarily sealed before centrifugation for 5 minutes at 500g to create the pellets. The seal was removed before the pellets were cultured in defined culture environments, e.g. 20% O_2 or 1% O_2. The pellet aggregates were released from the bottom of the wells after 24 hours of culture by aspiration and releasing 100ul of the medium. The medium was changed every other day to avoid acidification. The cultures were maintained for periods of up to 14 days.

Culture of other cell types

A human prostate cancer cell line (PC3) and a human chondrosarcoma cell lines (C28/I2, a gift from Mary Goldring; Cornell University, USA) were selected as positive controls for HIF-1α protein expression. The cell lines were cultured only in monolayer systems because of their aggressive growth. C28 cells were cultured in DMEM supplemented with 10% foetal calf serum, 100unit/ml penicillin G sodium and 100µg/ml streptomycin. PC3 cell line was cultured in RPMI-1640 containing the same supplements

as in DMEM above. The cell lines were subcultured twice a week owing to the brief time taken to attain confluence.

Culture under different oxygen conditions

Unless stated otherwise, tissue cultures were prepared and maintained at 37°C with different gas phase oxygen concentrations, 20% (using CO_2 incubator; Sanyo MCO-17AIC) and 1% O_2,(using O_2/CO_2 incubator; Sanyo MCO-18M), in the present of 5% CO_2 with the remaining gas being nitrogen.

Laboratory methods

1. **Total protein extraction from cell cultures**

Confluent monolayer cells (~500,000 cells) were washed twice in phosphate buffer saline (PBS). All steps were carried out at 0-4°C. Cell layer were scraped from flasks in RIPA buffer (PBS with 1% Igepal CA-630[Sigma], 0.5% sodium deoxycholate and 0.1% SDS) with freshly added 1% (v/v) protease inhibitor cocktail (Sigma P-8340) and transferred to a microcentrifuge tube. The suspension was homogenised using a syringe fitted with a 21 gauge needle and incubated for 1 hour on ice. The cell lysate was separated from sediment by centrifugation at 10,000g for 10 minutes at 4°C. The supernatant fluid containing cell lysate was collected and kept at -80°C until use.

2. Nuclear extraction

Confluent monolayer cells (~500,000 cells) were washed twice in PBS before being resuspended in a low salt buffer containing 10mM HEPES pH 7.9, 10mM KCl, 10mM EDTA, 1mM dithiothreitol (DTT), and 1% (v/v) protease inhibitor cocktail (Sigma P-8340) and incubated for 10 minutes on ice with gentle shaking. All steps were carried out at 0-4°C. The cellular suspension was then transferred to a microcentrifuge tube and centrifuged at 10000g for 10 minutes at 4°C to separate the soluble cytosolic fraction. The pellet was resuspended in a high salt buffer, containing 20mM HEPES pH 7.9, 10% glycerol, 400mM NaCl, 1mM DTT and 1% (v/v) protease inhibitor cocktail (Sigma P-8340). The suspension was homogenized by a Dounce homogeniser and incubated for 2 hours with gentle shaking and centrifuge at 15,000g for 10 minutes at 4°C. The supernatants containing the nuclear extract were collected and stored at -80°C until use.

3. Protein analysis

Protein measurement was performed using the Folin and Ciocalteau's phenol reagent with a method adapted from Lowry et al. (1951). 3% Na_2CO_3 in 0.2M NaOH and 1% $CuSO_4.5H_20$ in 2% Na-K tartrate were freshly prepared as solution A and solution B respectively. Two millilitre of solution B was added to 48ml solution A to become a working solution. Standards were prepared from bovine serum albumin at concentrations from 0 to 250μg/ml. Aliquots (200μl) of standards and samples were mixed well with the prepared working solution and allowed to stand for 10

minutes at room temperature. 50µl of the Folin and Ciocalteau's phenol reagent was then added and mixed. The samples were read after 10 minutes incubation at room temperature in a spectrophotometer at OD 640.

4. DNA measurement

Double stranded DNA was measured by Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen), which is a fluorescent nucleic stain. The assay is able to detect DNA in a range of 1-1000 ng/ml. Samples and DNA standards were diluted in Tris EDTA buffer and 100 µl of each sample and standard were loaded into a black 96-well microplate followed by 100µl of PicoGren® reagent. The plates were incubated for 5 minutes at room temperature before reading on a plate reader with excitation at 480nm and emission at 520nm.

5. DMMB assay

Tissue culture media and cartilage or papain digests were assayed for sulphated glycosaminoglycan using a modification of the 1,9-dimethylmethylene blue dye binding assay (DMMB)(Farndale et al. 1986). Chondroitin sulphate C (from shark cartilage) at 0-50µg/ml was used as standard. The assays were performed in duplicate using 96 well-plates. Samples or standard (40µl) were mixed with 200µl DMMB solution and read immediately at 525nm absorbance. The data were analysed by Ascent software (Thermo Labsystems).

6. Hydroxyproline assay

Collagen degradation was determined by measuring released hydroxyproline, which is found primarily in collagenous sequences and is therefore used as an indicator of collagen content. The assay was adapted from the method decribed by Cawston et al. (1999). In brief, oxidant solution and colour reagent (Ehrlich's reagent solution) were freshly prepared on each day of assay. Oxidant reagent consisted of 7% (w/v) chloramine T diluted 1:6 in assay buffer (57g sodium acetate, 42.74g tri sodium citrate, 5.5g citric acid and 400 ml propan-2-ol per litre water). Colour reagent was made from dimethylamino benzaldehyde (DAB) solution (in proportions of 2g DAB to 3 ml of 60% perchloric acid) diluted 1:5 in propan-2-ol. Samples were hydrolysed in 6M HCl at 110°C for 24 hours and then the hydrolysates were freeze-dried to remove the acid. The freeze-dried hydrolysates were reconstituted in distilled water and centrifuged at full speed for 10 minutes to separate insoluble material, as it interferes with the assay. Serial dilutions (1-10μg/ml) of hydroxyproline (Sigma) were used as standards. The assay was performed in 96 well-plates in duplicates. Both standards and samples (30μl) were mixed with 70μl diluent (1:3 diluted propan-2-ol in water), following by adding 50μl of oxidant reagent and 125μl of colour reagent. Plates were incubated at 70°C for 20 minutes and left to cool to room temperature before reading at 550nm absorbance. The data were analysed by Ascent software (Thermo Labsystems).

7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared according to the method of Laemmli (1970) on the Biorad mini-gel apparatus. Unless stated otherwise, 7% acrylamide resolving gels were used in most experiments. Samples were diluted in sample buffer (with addition of 5mg/ml DTT or β-mercaptoethanol at 1:20 dilution in reduced samples) and boiled for 4 minutes prior to electrophoresis. Gels were run within running buffer at 200V for approximately 45 minutes. The resolved proteins were either transferred for western blotting or stained with 0.2% Coomassie brilliant blue. The reagents that were used are shown in Table 2.1.

Table 2.1 SDS-PAGE and staining reagents

Reagent	Amount	Reagent	Amount
Resolving gel (7% acrylamide for 2 gels)		Stacking gel (4% acrylamide for 2 gels)	
30% acrylamide	3.11 ml	30% acrylamide	0.523 ml
1M Tris HCl pH 8.8	5 ml	1M Tris HCl pH 6.8	0.41 ml
10% SDS	0.133 ml	1% SDS	0.33 ml
H_2O	4.64 ml	H_2O	1.878 ml
1.5 % APS	0.433 ml	1.5% APS	0.167 ml
TEMED	17 µl	TEMED	7 µl
Sample buffer (1X)		Running buffer (500 ml; 10X)	
1M Tris HCl pH 6.8	4 ml	Tris	15 g
20% (w/v) SDS	7.5 ml	Glycine	72 g
Glycerol	5 ml	SDS	5 g
Bromophenol blue	300 µl	Coomassie stain (0.2%)	
H_2O	3.2 ml	Brilliant blue	1 g
Destain		Methanol	150 ml
Methanol	150 ml	Glacial acetic acid	50 ml
Glacial acetic acid	35 ml	H_2O	300 ml
H_2O	315 ml		

8. Western Blots

Proteins resolved by SDS-PAGE were electrotransferred (BioRad minoblot apparatus) to polyvinylidene fluoride (PVDF) membrane using buffer containing 250mM tris and 200mM glycine at 150 V for 1 hour. The blotted membranes were blocked by 5% skimmed milk in PBS with 0.5% Tween-20 (PBST) for 1 hour on a rocking platform. After being washed in PBST, the blots were probed by primary antibody diluted in PBST for 1 hour and then washed at least three times (10 minutes each) before applying secondary antibody and incubated for another 1 hour. The dilutions of primary and secondary antibodies and their substrates are shown in Table 2.2. For chemiluminescence, the blots were developed by enhanced chemiluminescent reagents (PIERCE) for 5 minutes before exposing to the x-ray films.

Table 2.2 Antibodies and substates used in Western blots

Primary antibody	Dilution	Secondary antibody	Dilution	Substrate
HIF-1α polyclonal antibody (Santa Cruz)	1:200	Anti-rabbit IgG conjugated with HRP*	1:50,000	Chemiluminescent reagents (PIERCE)
HIF-1α monoclonal antibody (BD transduction)	1:250	Anti-mouse IgG conjugated with HRP*	1:10,000	Chemiluminescent reagents (PIERCE)
Type II collagen (AVT6E3)**	1:10	Anti-mouse IgG conjugated with alkaline phophatase	1:5,000	SIGMA FAST™ BCIP/NBT****
GLUT-1***	1:2,000	Anti-rabbit IgG conjugated with alkaline phophatase	1:5,000	SIGMA FAST™ BCIP/NBT****

* Horse radish peroxidase
** Antibody was donated from Dr Anne Vaughan-Thomas (University of Liverpool, United Kingdom)
*** Antibody was donated from Dr S.A. Baldwin (University of Leeds, United Kingdom)
**** 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium

9. RNA extraction and Bioanalysing

Total RNA was extracted from samples using TRIzol® Reagent (Invitrogen). Cells in monolayer were scraped with 1 ml TRIzol® per 75cm^2 flask and transferred to a microcentrifuge tube. Cartilage explants were snap frozen and pulverised in liquid nitrogen cooled steel chambers (Braun Biotech Mikrodismembrator) and suspended in 1 ml TRIzol®. Chondrocyte pellet cultures were homogenised with 1 ml TRIzol® by using Molecular Grinding Resin and a small pestle (Web Scientific: 786-138PR). RNA was separated from TRIzol® by vigorous agitation with chloroform (200µl/1ml TRIzol®) and centrifugation at 12,000g for 10 minutes at 4°C. Clear supernatant was mixed with isopropyl alcohol and centrifuged at 12,000g at 4°C to precipitate RNA. RNA pellets were washed in 75% ethanol and left until almost dry and then redissolved with RNase-free water. RNA samples were purified through RNeasy columns with DNaseI (Qiagen), according to the manufacturers' instructions to remove genomic DNA and finally eluted in water. RNA quantification and qualification were measured by Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturers' instructions.

10. cDNA synthesis

cDNA copies were made from 1-13µg RNA with RIN (RNA integrity number) 6 or above, except in the reference gene selection study in which a fixed amount of 1.5µg RNA was used. In each sample, 2µl Mastermix I [1:1 mixture of 500µg/ml oligo (dT) primer (Promega) and 10mM dNTPs

(Invitrogen)] was added into 20µl RNA solution. RNA mixtures were incubated at 65°C for 5 minutes before 7µl of Mastermix II [4µl of 5x First strand buffer, 2µl of 0.1M DTT; both supplied by Invitrogen, and 1µl of RNasin (Promega)] was added in to each sample. The mixtures were incubated at 42°C for 2 minutes prior to adding 1µl SuperScript II (Invitrogen) and incubated at the same temperature for 50 minutes. The reaction was stopped by incubation at 70°C for 15 minutes. The synthesized cDNA samples were kept at -20°C until use.

11. Primer design and validation

Equine RNA and DNA sequences were sourced from the NCBI database (http://www.ncbi.nlm.nih.gov/). If the equine sequence was unavailable then a multiple species alignment was carried out (www.ebi.ac.uk/Tools/clustalw/) and primers designed where there was sequence homology. Human exon boundaries were identified using the Ensembl Genome Browser (http://www.ensembl.org/index.html), and where possible, primers were designed to cross predicted exon boundaries. The primers were designed based on the above sequences using Primer Express (Applied Biosystems) software or Roche primer design (https://www.roche-applied-science.com/) or Primer3 software (http://primer3.sourceforge.net/). Primer efficiencies were validated using a standard curve derived from equine tendon cDNA (a ten fold dilution series with five measuring points).

12. Real-time (quantitative) polymerase chain reaction (qPCR)

PCR reactions were performed in 386-well PCR plates (Applied Biosystems). In each well, 4.6µl cDNA was mixed with 0.1µl water (molecular biology grade), 0.3µl of 300 nM forward and reverse primers mixture and 5µl SYBR® Green PCR master mix. The plates were securely sealed with Optical adhesive covers (Applied Biosystems) and processed in a 7900HT Fast Real time PCR system (Applied Biosystems) using standard amplification conditions. The initial denaturation was performed at 95°C for 10 minutes. Forty cycles of amplification were performed. Each cycle involved a denaturation step of 15 seconds at 95°C and followed by 60 seconds primer annealing at 60°C. After the last cycle, a dissociation curve was performed at 95°C for 15 seconds and 60°C for 15 seconds in order to ensure one product of amplification. Each reaction was run in triplicate. PCR products were measured and analysed by SDS software (v 2.2.1. Applied Biosystems) and then normalized to previously-selected reference genes (see **Chapter IV**).

Chapter III

GLUCOSE TRANSPORTATION IN CHONDROCYTES UNDER HYPOXIA

This work has been accepted for publication in the Journal of Orthopedic Research (see Appendix)

Introduction

Articular cartilage is an avascular tissue. Nutrients and oxygen are delivered by diffusion from blood vessels of the underlying bone and from the synovial fluid (Lee and Urban 1997). The oxygenation gradients within the tissue have been estimated to be 6-10% at the articular surface to less than 1% O_2 at the deepest layer (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971; Silver 1975; Kiaer et al. 1988; Ferrell and Najafipour 1992). However, chondrocytes appear to adapt very well to oxygen and nutrient limited environments (Rajpurohit et al. 1996). Both anabolic activity and cell survival of chondrocytes were maximized when they are cultured *in vitro* under low oxygen tensions compared to normoxic and anoxic conditions (Grimshaw and Mason 2000; Hansen et al. 2001; Domm et al. 2002; Schneider et al. 2004).

The main source of metabolic energy in chondrocytes is glucose which is taken up by a family of substrate specific membrane proteins known as glucose transporters (GLUTs). GLUT isoforms differ in their substrate

specificity, tissue distribution and cellular localisation. Articular chondrocytes and intervertebral disc cells express a number of glucose transporter proteins including GLUT1, GLUT3 and GLUT9 (Mobasheri et al. 2002 b). GLUT1 and GLUT3 are hypoxia responsive isoforms (Semenza 2001 b) which are regulated by the transcription factor and oxygen sensor protein, hypoxia-inducible factor 1 (HIF-1)(Mobasheri et al. 2005 a; Richardson et al. 2008).

Chondrocytes generate energy mainly by anaerobic glycolysis (Lee and Urban 1997), which produces 18-19 times less ATP per molecule of glucose than aerobic respiration (Ahlqvist 1984). Therefore, chondrocytes must be able to manage glucose transport and metabolism effectively to provide sufficient energy within the constraints of what is available in cartilage. Recent studies (Mobasheri et al. 2006), (Zhang et al. 1999) have shown that chondrocytes are capable of maintaining their metabolic balance when deprived of glucose or exposed to the hypoxia mimetic cobalt chloride ($CoCl_2$). $CoCl_2$ induces hypoxic conditions by inhibiting iron-dependent HIF-prolyl hydroxylases (Déry et al. 2005) so that the ubiquination and proteosome degradation of HIF-1α are blocked.

Osteoarthritis (OA) leads to progressive and permanent loss of articular cartilage. Joint injury, the major cause of OA in the horse (McIlwraith 2002), leads to synovial effusion and increasing vascularisation in response to inflammation and healing processes and this may disturb or alter the

oxygen supply within the synovial environment (Blake et al. 1989). Osteochondritis dissecans (OCD) is categorised as a developmental orthopaedic disease, involving a failure of endochondral ossification which is associated with a failure of vascular invasion from the subchondral bone and leads to development of cartilage lesions that can become detached at a later date (McIlwraith 2002). The ischemia-reperfusion from the neo-vascularisation process in traumatic joints before OA development (Blake et al. 1989) and the ischemic necrosis of the subchondral bone during OCD formation may cause fluctuations in oxygen levels and result in metabolic imbalance by disturbing glucose transport.

The aim of this study was to test the hypothesis that chondrocytes are able to adapt their energy metabolism under hypoxia, using low oxygen (1% O_2) or exposure to a hypoxia mimetic (cobalt chloride). We also investigated whether expression of a key hypoxia responsive glucose transporter (GLUT1) varied in normal cartilage compared to degenerative cartilage, such as found in osteoarthritis (OA) and osteochondritis dissecans (OCD).

Materials and Methods

Reagents

All tissue culture reagents were from Invitrogen. 2-Deoxy-D-[2,6-^{3}H] glucose and NACS104 aqueous scintillation cocktail were from Amersham Biosciences. Polyclonal antibodies against GLUT1 were donated by Dr

S.A. Baldwin (University of Leeds, UK). The GLUT1 antiserum was developed in rabbits against the C-terminus of rat-GLUT1 (residues 477-492). We have previously confirmed that this GLUT1 antibody recognizes GLUT1 across a diverse number of mammalian species including rat, human, dog, sheep and horse (Mobasheri et al. 2005 b). Anti-rabbit IgG conjugated with FITC was obtained from Sigma.

Cartilage source

Normal equine articular cartilage was obtained from horses aged between 2-12 years old, euthanased for clinical reasons at the Philip Leverhulme Equine Hospital, University of Liverpool. Normal, OA and OCD cartilage explants were also collected and processed for mRNA isolation.

Chondrocyte isolation and culture

Cartilage for chondrocyte cultures was obtained from healthy horse joints only. Each sample was rinsed and chondrocytes were isolated to culture in monolayers (see **Chapter II**). All experiments were performed on first passage cells.

Chondrocyte proliferation and viability

Equine chondrocytes were expanded in monolayer and subcultured to passage one in $25cm^2$ tissue culture flasks. The cultures were divided for two experiments. A set of cultures was cultured either under 1% or 20% O_2 from the first day. Chondrocytes were washed with PBS and trypsinised before they were counted. Each culture was counted daily for seven days using a haemocytometer. Another set of chondrocyte monolayers was

maintained under 20% O_2 until nearly confluent and then treated with 1% O_2 or 20% O_2 with or without 75μM $CoCl_2$ for 48 hours. The viability and numbers of chondrocytes was assessed using trypan blue dye exclusion and counted in a haemocytometer. The study was performed in triplicate using three different donors.

Deoxy-D-[2,6-^{3}H] glucose uptake

Net glucose transport was determined by measuring the uptake of non-metabolisable deoxy-D-[2,6-^{3}H] glucose into equine chondrocytes in 24-well plates in the presence and absence of the glucose transport inhibitor, cytochalasin B (20μM). Monolayer chondrocyte cultures were preconditioned in 20% or 1% O_2 with and without 75μM $CoCl_2$ for 48 hours and rinsed with PBS three times. The uptake of deoxy-D-[2,6-^{3}H] glucose (0.5 μCi/ml culture medium) was assayed for 2 hours at 37°C in modified DMEM lacking glucose, pyruvate and serum. The 24-well plates were washed three times with ice-cold PBS and the chondrocytes lysed with a cell lysis solution consisting of 0.5 % (v/v) SDS and 0.5 % (v/v) Nonidet in PBS. Aliquots (450μl) of the cell lysis solution were mixed with 3.55ml of aqueous scintillation cocktail (NACS104; Amersham Biosciences) and counted in a scintillation counter (Packard). The remaining 50μl aliquots of the cell lysis solution were used to determine total cell protein content using the Lowry protein assay (Lowry et al. 1951) and 2-deoxyglucose uptake was normalized to total cell protein. All uptake experiments were carried out in triplicate and repeated under identical

conditions (at least three times) and the data are presented as percentage change in total deoxy-D-[2,6-^{3}H] glucose uptake.

GLUT-1 Western blot

A whole cell lysate, extracted from first passage equine chondrocytes using RIPA buffer, was run on SDS-PAGE using 10% acrylamide and transferred to a PVDF membrane. The membrane was probed with GLUT1 antibody for 2 hours and with alkaline phosphatase conjugated anti-rabbit IgG for 1 hour at room temperature. SIGMA FAST^{TM} BCIP/NBT was used as the substrate. A secondary antibody control was also performed.

Fluorescence activated cell sorting (FACS) analysis of GLUT1 expression

Nearly confluent first passage monolayer equine chondrocytes were maintained in 25 cm^2 tissue culture flasks under 20% O_2 or 1% O_2 for 48 hours. After being trypsinised, the isolated cells were transferred to 1.5 ml Eppendorf tubes and washed using PBS containing 1% FCS followed by centrifugation at 1400 rpm for 5 minutes. Chondrocytes were fixed with 3.7% paraformaldehyde for 10 minutes at ambient temperature and permeabilised with 0.05% Triton X 100 in PBS for 10 minutes at 4°C. The cells were incubated with anti-GLUT-1 polyclonal antibody at the dilution of 1:600 in PBS, followed by goat anti-rabbit IgG conjugated with FITC at 1:40 in PBS, each for 30 minutes at 37°C in light protective containers. Between each step the washing was performed as described above. The final washing and resuspension were done using PBS before transferring to

5 ml (12 x 75 mm) polystyrene round-bottom tubes (Becton Dickinson) and analysis by FACScan (Becton Dickinson) by gating 10,000 events. The morphological profile of the cells was detected by combining forward light scatter (FSC) and side light scatter (SSC). The detectors were set as follows: FCS (threshold): 200; FSC: E-01; SSC: 319 and FLI: 408 and the parameters were: FSC-H: 5.0; SSC-H: 1.2 and FL-1 H: log. The autofluorescence, expressed from unstained chondrocytes and the non-specific staining from secondary antibody were subtracted in each experiment (Fig 3.1). The data were analysed by WinMDI 2.8 (free downloadable software; http://facs.scripps.edu/software.html) to obtain the number of cells expressing fluorescence and mean fluorescent intensity (MFI) in each sample.

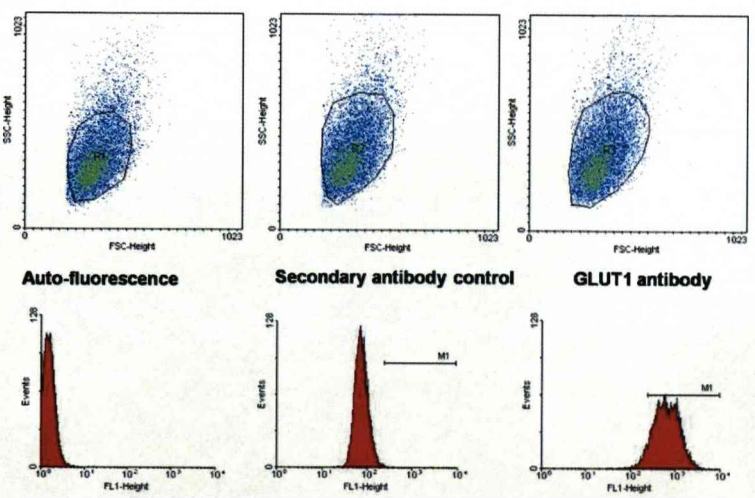

Fig 3.1 FACS Density plots and histograms generated by WINMDI 2.8 software. The top panel shows density plot of 10,000 chondrocytes and gated regions. The bottom panel shows fluorescence events of gated areas. The areas cleared from interference of auto-fluorescence and secondary antibody are marked (M1).

RNA and cDNA preparation and real-time PCR to detect changes in the levels of GLUT1 expression

Total RNA was extracted from chondrocyte monolayers and cartilage explants from normal, OA and OCD horses using the method described in **Chapter II**. The mRNA expression of GLUT-1 was assessed in monolayer cultures (n = 3) of equine chondrocytes exposed to 20% O_2 or 1% O_2 with and without 75 μM $CoCl_2$ for 24 hours and the cartilage explants from normal (n = 6), OA (n = 8) and OCD (n = 5) horses. The qPCR was performed with the following primers designed by Primer3: forward primer; 5'-AGCAGCCTGTGTACGCCAC-3' and reverse primer; 5'-CTCGTTCCACCACAAACAGC-3'. PCR products were measured and normalized by the selected reference genes.

Reference gene selection

The candidate reference genes and their primers are shown in Table 3.1. They were selected on the basis that they belong to different functional classes. Some of the primer sequences were from a reference gene study using equine skin (Bogaert et al. 2006). The rest of the candidate reference gene primers using in this study were self designed using the same method as in primer design and validation (see**Chapter II**). Expression of all seven candidate reference genes were analysed across the chondrocyte monolayer cultures (n=5) exposed to 20% O_2 or 1% O_2 with and without 75 μM $CoCl_2$ or 100 ng/ml IL-1β for 24 hours. Another analysis was done across normal (n = 6), OA (n = 8) and OCD (n = 5) cartilage to investigate the expression

of five candidate reference genes (HPRT-1, TBP, GAPDH1, GNB2L1 and 18S). The most stable pair of genes was identified using downloadable software; GeNorm (**http://medgen.ugent.be/~jvdesomp/genorm/**) and Normfinder (**http://www.mdl.dk/publicationsnormfinder.htm**), according to the software instructions.

Table 3.1 Candidate reference gene list

Symbol	Gene name	Accession Code	primers 5'-3'	Efficiency on equine tendon	References
GAPDH (1)	Glyceraldehyde-3-phosphate dehydrogenase	AF157626	Fw: GCATCGTGGAGGGACTCA Rv: GCCACATCTTCCCAGAGG	-3.317	Roche
GAPDH(2)	Glyceraldehyde-3-phosphate dehydrogenase	AF157626	Fw: TGACCCCCTAACATATTGAGAGTCT RV: GCCCCTCCCCTTCTTCTG	-3.139	Primer Express
ACTB	Actin, beta	AF035774	Fw: CCAGCACGATGAAGATCAAG Rv: GTGGACAATGAGGCCAGAAT	-3.333	Bogaert et al, 2006
TBP	TATA box binding protein	NM_001075742	Fw: TGCTGCTGTAATCATGAGGGTAA Rv: TCCCGTGCACACCATTTTC	-3.54	Primer Express
HPRT-1	Hypoxanthine phosphoribosyltransferase I	AY372182	Fw: GGCAAAACAATGCAAACCTT Rv: CAAGGGCATATCCTACGACAA	-3.35	Bogaert et al, 2006
18S	Ribosomal protein, 18S	AJ311673	Fw: GGCGTCCCCCAACTTCTTA Rv: GGGCATCACAGACCTGTTATTG	-3.241	Primer Express
GNB2L1	Guanine nucleotide binding protein	NM_006098	Fw: CCTTGTGCTTCAGTCCCAAT Rv: CAATGATCTTGCCCTTCAAGT	-3.241	Roche

Statistical analysis

Paired *t*-test and one way ANOVA with Dunnett's multiple comparison test was performed by GraphPad Prism software (v.4.01). Paired *t*-test was used to compare chondrocyte proliferation under normoxia and hypoxia. One way ANOVA was used for other analyses.

Results

Chondrocyte proliferation and viability

Chondrocyte monolayer cultures maintained under normoxia (20% O_2) and hypoxia (1% O_2) showed similar cell numbers in the first three days of culture. After day 4, there was net growth of cells and the increase of

chondrocytes numbers in normoxia was more than in hypoxia but it was not significantly (p>0.05) increased (Fig 3.2a). Chondrocytes survived very well after treatments with different oxygen tensions and $CoCl_2$. The viabilities were almost 100% of the total chondrocytes in every treatment (Fig 3.2b).

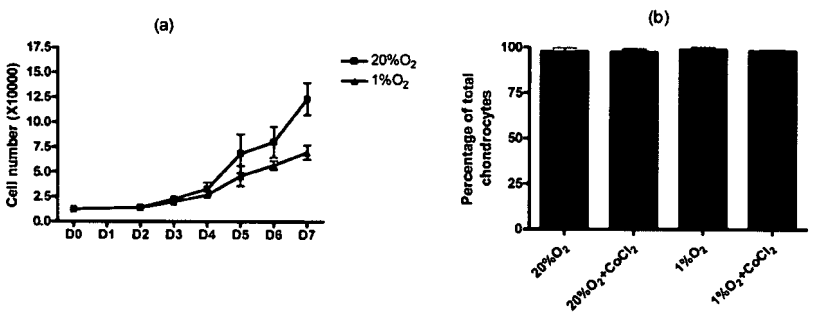

Fig 3.2 Numbers and viability of equine chondrocytes under hypoxia and normoxia. (**a**) Numbers of chondrocytes at the first passage from day 0 to day 7 under 20% and 1% O_2 (**b**) Chondrocytes viability after being cultured under normoxic and hypoxic conditions with or without 75μM $CoCl_2$. Data represents in percentage of total chondrocytes. The error bars represent SEM.

Deoxy-D-[2,6-^{3}H] glucose uptake

To determine potential alterations in glucose transport across equine chondrocyte membranes in response to differential oxygen levels, the effects of normoxia (20% O_2), hypoxia (1% O_2) and a hypoxic mimetic (75μM $CoCl_2$) were analysed on the uptake of deoxy-D-[2,6-^{3}H] glucose in normal equine articular chondrocytes. The average uptake by the control group (20% O_2) was set as 100%. Both hypoxia and $CoCl_2$ significantly increased glucose transport in equine chondrocyte cultures (Fig 3.3) (p<0.05). Moreover, the combination of hypoxia and $CoCl_2$ had an additive effect on glucose uptake comparing to hypoxia or $CoCl_2$ alone (Fig 3.3)

(p<0.01). Cytochalasin B was effective in inhibiting deoxy-D-[2,6-^{3}H] glucose uptake (p<0.01).

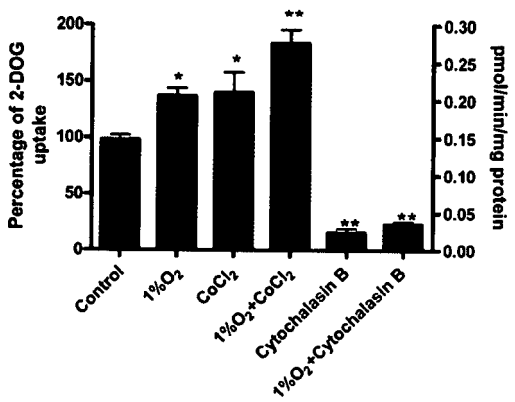

Fig 3.3 Effect of hypoxia (1% O_2), cobalt chloride (75μM) and the combined treatment on 2-deoxy-D-[2,6-^{3}H] glucose uptake by first passage equine articular chondrocyte monolayer (n=3). Results represent percentage glucose uptake by comparison to the control group (20% O_2) (Average uptake in the control group was taken as 100%, which is approximately 0.123pmol/min/mg protein). The error bars represent SEM.* p<0.05; ** p<0.01.

GLUT1 western blot

The expression of GLUT1 protein by equine chondrocytes was demonstrated by Western blotting using rabbit anti-GLUT1 antibody. Two immunoreactive bands were detected at around 45-50 kDa (Fig 3.4). The difference of the two GLUT1 appeared on the blot was probably due to glycosylation (Birnbaum et al. 1986; Kasaniki et al. 1987). A secondary antibody control blot showed no non-specific reaction of the anti-rabbit IgG antibody.

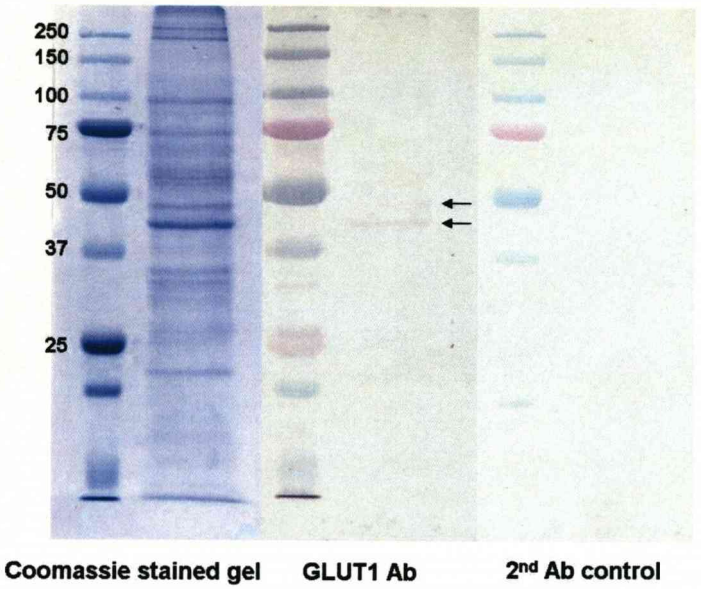

Fig 3.4 Immunoblot of GLUT1 (arrows) from a whole-cell lysate of first passage equine chondrocytes. The blot is shown in parallel with a Coomassie stained gel and a secondary antibody only (no primary antibody used) control blot run with the same sample.

Fluorescent activated cell sorting (FACS) analysis

Examples of FACS density plots and histograms of each treatment are shown in Fig 3.5. The analysis indicated that approximately 90% of all groups of chondrocytes expressed GLUT1 membrane protein (Table 3.2); however, statistical analysis revealed no difference in the number of chondrocytes that bound the GLUT1 antibody among all different oxygen tension and $CoCl_2$ treatments. Further analysis showed that the intensity of GLUT1 expression in chondrocytes exposed to 1% O_2, $CoCl_2$ and the combination of those 2 treatments was significantly increased (Fig 3.6)($p<0.05$) indicating that in response to hypoxic treatment, cells had increased numbers of GLUT1.

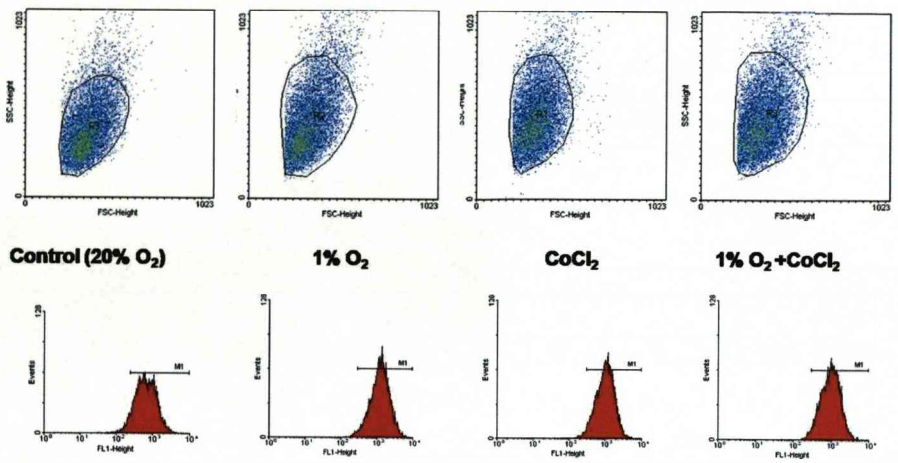

Fig 3.5 Examples of FACS density plots and histograms in each chondrocyte treatment (M1 = marker of area after subtraction of possible non-specific fluorescence of secondary antibody and chondrocyte auto-fluorescence).

Table 3.2 Mean ± standard error of number of chondrocytes that expressed GLUT1 (from 10,000 gated cells)

Treatment	Mean ± SE
Control	8997.33 ± 415.5
Hypoxia (1% O_2)	9300.78 ± 445.1
75 µM $CoCl_2$	9427.89 ± 246.82
Hypoxia + $CoCl_2$	9419.22 ± 285.50

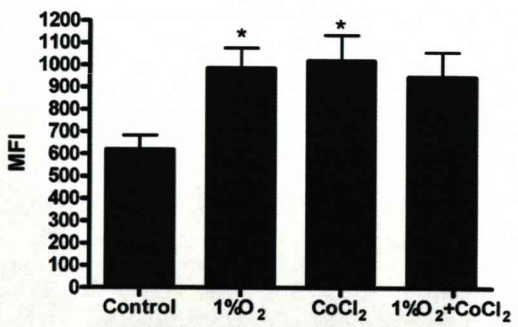

Fig 3.6 Changes in equine chondrocytes GLUT1 protein expression assessed by FACS analysis using first passage equine chondrocytes in monolayer after exposure of to hypoxia (1% O_2), cobalt chloride (75µM) and the combined treatment for 48 hours. The results indicate mean fluorescent intensity (MFI) per chondrocyte. The experiment was done with 3 different chondrocyte donors. The error bars represent SEM.* $p<0.05$.

Reference gene selection for qPCR analysis

The real-time PCR assays were designed to detect and quantify expression of commonly used reference genes (Table 3.1). The stability of gene expression across different samples was analysed using GeNorm and Normfinder software. The stability values and the ranking of the candidate genes from both forms of analysis in the study with chondrocyte cultures and a study of normal versus diseased cartilage study are shown in Table 3.3. Although the ranking of the candidate genes was somewhat different between the two programs, HPRT-1 and TBP were identified, in both studies and both analyses, to be the most stably expressed pair of genes and were selected for use as the reference genes for the rest of this study. Normfinder also indicated GAPDH(1) as the best single reference gene in the normal-disease cartilage tissue study.

Table 3.3 Stability value and ranking of candidate reference gene expression

		Normfinder			GeNorm Stability	
Experiment	Gene	Stability value	Rank	Gene	Value	Rank
Equine chondrocyte cultures	HPRT-1*	0.195	1	HPRT-1	0.244047225	1
	TBP*	0.204	2	TBP	0.244047225	2
	GNB2L1	0.244	3	GAPDH (1)	0.54765329	3
	18S	0.283	4	GAPDH (2)	0.560551071	4
	GAPDH (1)	0.336	5	GNB2L1	0.602052909	5
	GAPDH (2)	0.378	6	18S	0.650220526	6
	ACTB	0.451	7	ACTB	0.712836738	7
Normal and diseased cartilage explants	GAPDH (1)	0.048	1	HPRT-1	0.643906	1
	HPRT-1*	0.055	2	TBP	0.643906	2
	TBP*	0.058	3	GAPDH (1)	0.728417	3
	GNB2L1	0.117	4	GNB2L1	0.890601	4
	18S	0.141	5	18S	1.248013	5

* The best combination of two genes by Normfinder

GLUT1 mRNA expression

The relative expression of GLUT1 mRNA was quantified by real-time PCR assay using SYBR® Green. The Ct values of all samples were normalised with the mean Ct values of the selected reference genes, which were HPRT-1 and TBP. There was an up-regulation of GLUT1 expression in chondrocytes exposed to 1% O_2 and 75 μM $CoCl_2$: however; only a combination of both treatments showed a statistically significant increase *($p<0.05$)* (Fig 3.7a).

In contrast, examination of GLUT1 mRNA expression in cartilage tissue identified that in OA cartilage its abundance was significantly decreased (Fig 3.7b)*($p<0.01$)* compared to normal cartilage. In OCD samples, expression of GLUT1 was also decreased but this reduction did not reach statistical significance. In summary, this data indicated some suppression in transcription of GLUT1 in pathological cartilage.

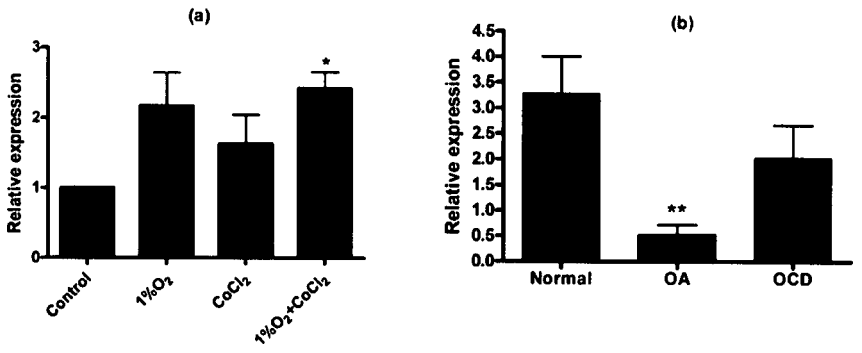

Fig 3.7 GLUT1 mRNA expression (**a**) study of first passage equine chondrocyte monolayers after exposure to normoxia (20% O_2), hypoxia (1% O_2), and/or 75μM cobalt chloride for 24 hours using 5 different donors and (**b**) study in normal (n= 6), osteoarthritis (OA) (n=8) and osteochondritis dissecans (OCD) (n= 5) cartilage explants. The analysis was done in triplicate. The error bars represent SEM.* $p<0.05$; ** $p<0.01$.

Discussion

In this study of chondrocyte physiology we observed that equine chondrocytes survived very well under hypoxia in the presence of a hypoxia mimic environment confirming a result from previous report which showed chondrocyte viability under hypoxia for up to 11 days (Schneider et al. 2004). An enhanced transportation of glucose by equine chondrocytes in a low oxygen environment is supportive of previous studies in equine chondrocytes using $CoCl_2$ (Mobasheri et al. 2006). This may suggest either an attempt to maintain the basal cellular energy status or increasing metabolic demand of the chondrocytes in response to hypoxic conditions. However, Schneider and colleaques (2004) demonstrated that ATP content of equine chondrocytes is stable under various oxygen concentrations. Therefore, it is unlikely that chondrocytes increase their energy demand under hypoxia.

The inhibition of glucose uptake by cytochalasin B indicates that the glucose uptake is mediated by sodium-independent glucose transporters (McNulty et al. 2005). Glucose transporter 1 (GLUT1) is known to be the most abundant GLUT expressed by most cell types, including chondrocytes and is considered to function as a 'house keeping' glucose transporter. In this role, GLUT1 maintains basal glucose uptake for metabolic reactions (Mobasheri et al. 2002 a). GLUT1 is also reported to be a 'hypoxia-responsive' glucose transporter in various tissues (Vannucci et al. 1996; Behrooz and Ismail-Beigi 1997; Zhang et al. 1999), especially

in cancer cells which collectively exhibit the Warburg effect (reviewed by Airley and Mobasheri 2007). This observation was confirmed by our investigation of chondrocyte GLUT1 protein expression. We found that although most of the chondrocytes examined expressed some GLUT1 protein, the physical (1% O_2) and chemical ($CoCl_2$) hypoxia signals significantly increased the amount of GLUT1 that is expressed on each chondrocyte.

However, the glucose uptake results were not fully concordant with the GLUT1 protein expression data, especially in chondrocytes exposed to a combination of hypoxia and $CoCl_2$. While glucose uptake showed an additive effect of the combined hypoxia treatment, GLUT1 expression was at the same level as individual 1% O_2 and $CoCl_2$ treatments, suggesting the involvement of other glucose transporters. There is a strong possibility that other glucose transporters such as GLUT3 and GLUT9 are also involved in this response and are regulated by hypoxia as their expression has been confirmed in human chondrocytes (Shikhman et al. 2001; Mobasheri et al. 2002 b). GLUT3 is known to be the high affinity glucose transporter (Mobasheri et al. 2002 b) and could have a role in regulating glucose transport under such stimuli where chondrocytes have a very high metabolic demand to maintain their function. Furthermore, GLUT3 has been found to be upregulated in a number of hypoxic tumours along with GLUT1 (reviewed by Airley and Mobasheri 2007).

We also investigated the gene expression of GLUT1, in response to low oxygen tension and the presence of cobalt chloride, using quantitative, real-time PCR. While GLUT1 protein expression was significantly higher in hypoxic conditions, GLUT1 mRNA expression whilst elevated, did not reach statistical significance. The smaller response in GLUT1 mRNA expression to hypoxia or $CoCl_2$ stimulation suggested that increasing the expression of GLUT1 gene may not be the main mechanism used by chondrocytes to regulate glucose metabolism under low oxygen environment; other mechanisms may be involved including post-translational regulation (i.e. recruitment and activation of pre-existing GLUT1 transporters). Zhang and colleagues (Zhang et al. 1999) have reported that the acute response to hypoxia is mainly mediated by enhanced function of the existing glucose transporters, especially GLUT1, while the regulation at transcriptional level of GLUT1 is found to increase in chronic hypoxia. However, our data did identify that the combination of hypoxia and $CoCl_2$ treatment caused a reproducible and significant increase in GLUT1 mRNA levels, indicating transcription of GLUT1 can be significantly increased acutely, dependent on the relevant stimuli.

In the second part of our study, we focused on the transcription of the GLUT1 gene in equine joint diseases. We compared GLUT1 expression in normal cartilage and cartilage derived from joints with either OA or OCD. Interestingly, our findings indicated decreased GLUT1 transcription in OA

and OCD specimens with a greater reduction and statistical significance in the OA samples, suggesting compromised glucose metabolism especially in OA chondrocytes. The observed reduction of GLUT1 transcription may be a consequence of fluctuations in oxygenation caused by systemic effects or disturbances in the vascular supply during pathological processes (Blake et al. 1989). However, HIF-1, the key protein linking oxygen tension and GLUT1 expression in equine chondrocytes has not yet been investigated in this species. Although there is evidence of increased HIF-1α and GLUT1 protein levels in human OA cartilage, possibly in order to compensate for the increase in ATP demand (Pfander et al. 2005), we identified downregulation of equine GLUT1 mRNA in equine OA cartilage suggesting failure to maintain an energy supply in equine degenerative cartilage.

Conclusion

Hypoxia and the osteoarthritic process have significant effects on glucose transport and metabolism in chondrocytes. Hypoxia increases the rate of glucose uptake and regulation of GLUT1 transporters whilst OA and OCD lead to a reduction in transcription of GLUT1 mRNA. These data may indicate a failure of chondrocytes in OA cartilage to maintain metabolic and structural glucose levels in joint disease.

Acknowledgements

We would like to special thank Professor Andrea Varro, Division of Physiology, School of Biomedical Sciences, University of Liverpool for scintillation counter facilities, Dr Dylan Clements for reference gene analysis and Miss Sarah Taylor for reference gene primer design and validation.

Chapter IV

EQUINE CARTILAGE DEGRADATION AND CHONDROCYTE CATABOLISM UNDER HYPOXIA

Introduction

Articular cartilage in the adult is an anatomically avascular tissue deriving oxygen by diffusion from synovial fluid and the subchondral bone (Lee and Urban 1997). The oxygenation gradient as low as 1% exists within healthy articular cartilage (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971; Silver 1975; Kiaer et al. 1988; Ferrell and Najafipour 1992); hence, chondrocytes are likely to live physiologically in a microenvironment that is hypoxic relative to other tissues. Functional adaptations of chondrocytes to hypoxia have been reported (Schipani et al. 2001; Pfander and Gelse 2007). A key protein in this adaptation is hypoxia inducible factor-1 (HIF-1) which has been shown to be involved in maintaining energy generation and cellular survival of hypoxic chondrocytes (Rajpurohit et al. 1996). It has also been reported that the anabolism and survival of chondrocytes is maximal when they were cultured *in vitro* under low oxygen tension compared to normoxic and anoxic conditions (Grimshaw and Mason 2000).

Although studies have provided evidence for the beneficial effects of hypoxia on cartilage matrix production (Grimshaw and Mason 2000; Murphy and Sambanis 2001 b; Malda et al. 2004 a), there is little information regarding its effects on cartilage matrix catabolism. One study using IL-1 stimulated bovine chondrocytes demonstrated that alterations in transcription levels of genes associated with cartilage matrix production and catabolism degeneration occurred under hypoxic and reoxygenation stress conditions (Martin et al. 2004).

Catabolism of cartilage extracellular matrix, which is mainly comprised of proteoglycan and type II collagen, leads to a biomechanically inferior tissue, and causes pathological changes such as in osteoarthritis. Although proteoglycan can be rapidly replaced, collagen loss may be irreversible (Eyre 2004). The degradation of cartilage matrix is mediated largely by members of the matrix metalloproteinases (MMPs) family of degradative enzymes. They are secreted in latent form and have a role in degradation of cartilage matrix upon activation (Nagase and Woessner 1999). The regulation of MMP activity can be controlled at the level of synthesis, proenzyme activation and inhibition of activated enzymes by tissue inhibitors of metalloproteinases (TIMPs). Proinflammatory cytokines have been known to induce cartilage catabolism (McInnes and Schett 2007) through up-regulation of these enzymes. *In vitro*, using porcine and bovine cartilage, it has been shown that interleukin-1 (IL-1) and tumour necrosis

factor alpha (TNF-α) can drive this degenerative process particularly when combined with oncostatin M (OSM) (Cawston et al. 1999; Morgan et al. 2006).

In the present study, we aimed to examine the relationship between oxygen concentrations and equine cartilage catabolic processes by assessment of gene expression, enzyme production and activation and also matrix degradation. We also report a variation of cytokine response in equine articular cartilage in comparison to that reported in other species, which could be useful information for further cartilage degradation studies using equine tissue.

Materials and methods

Reagents

All human recombinant cytokines were from Peprotech. MMP-13 fluorogenic substrate was from Calbiochem. SYBR® Green PCR master mix was from Applied Biosystems.

Cartilage degradation model

Normal equine (age 4-25 years old) cartilage was rinsed and cultured as cartilage explants (see **Chapter II**). After 24 hours of being cultured, cartilage explants were divided into 5 groups of treatments, which were untreated control; TNFα; IL-1β; OSM and the combination of IL-1β and OSM, with a concentration of 20ng/ml of each human recombinant cytokine. Cultures were maintained either under 20% or 1% oxygen for 5

days in the short-term experiments (n=3), 4 weeks in the long-term experiments (n=3), and 24 hours for mRNA studies (n=3). The experiments were performed in quadruplicate for each horse.

Proteoglycan degradation

Cartilage explant culture media were collected daily in short-term experiments and weekly in long-term experiments. The remaining cartilage was digested with 1mg/ml papain in 0.1 M sodium-phosphate buffer (pH 6.5), containing 5mM N-acetyl cysteine and 5mM EDTA, at 65°C overnight. Media and papain-digests were assayed for sulphated glycosaminoglycan using the DMMB assay (see **Chapter II**). The data were analysed by Ascent software (Thermo Labsystems). The results are shown as percentage of total GAGs in each explants sample.

Collagen degradation

The papain-digests and tissue culture media collected from the long-term experiment were analysed for collagen release using the hydroxyproline assay (see **Chapter II**). The data were analysed by Ascent software (Thermo Labsystems). The results are shown as percentage of total hydroxyproline present in the cartilage explant.

MMP-13 fluorogenic substrate assay optimisation

The MMP-13 enzymatic activity assay was optimised using serial dilutions of active MMP-13 containing media from a canine monocyte cell line (DH82) (donated by Miss Natalie Gabriel) stimulated with 1μg/ml lipopolysaccharide (LPS) for 18 hours. The FCS was tested for its

interference with the assay by using medium containing 0%, 1% or 10% FCS. Two concentrations (10μM and 100μM final concentration) of the MMP-13 fluorogenic substrate peptide (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2) (Knäuper et al. 1996 a), were used for substrate optimisation. The plate was read every 30 minutes up to 240 minutes to define the best reading point (see MMP-13 assay below for the assay method).

MMP-13 fluorogenic assay

MMP-13 enzymatic activity was assessed using the MMP-13 fluorogenic substrate (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2) (Calbiochem), which is preferentially cleaved by MMP-13 (Knäuper et al. 1996 a). Medium samples (40μl) from equine cartilage (n=2) explant cultures were incubated in black 96 well-plates with assay buffer (50μl) (50 mM HEPES, 200 mM NaCl, 1 mM CaCl2, 0.01% (v/v) Brij-35, pH 7.3) and a final substrate concentration of 10μM. Serial dilutions of active MMP-13 containing serum-free media from a canine monocyte cell line (DH82) were included on each plate to ensure a linear relationship between MMP-13 activity and substrate cleavage over the required range. Aliquots of DMEM were also included in order to determine the assay background. In order to obtain values for total (latent and active) MMP-13 levels in the explants medium, activation of the latent enzyme was performed by incubation with 0.5mM *p*-aminophenylmercuric acetate (APMA) at 37°C for 1 hour prior to addition of the substrate. Both active and total MMP-13 generated fluorescence were measured (FLx800 microplate reader; Bio-

TEK Instrument Inc.), adjusting the excitation wavelength to 325 nm and emission to 393 nm after incubation of samples with substrate for 3 hours at 37°C. The data were analysed by KCjunior software (Bio-TEK) and represented as the percentage of each control group. The assay was performed only with samples taken at week 3, where the most significant collagen release was identified.

Quantitative Polymerase Chain Reaction (qPCR)

Twenty-four hours cytokine treated and the control cartilage explants were snap frozen and pulverised in liquid nitrogen cooled steel chambers (Braun Biotech Mikrodismembrator) before RNA and cDNA preparation (see **Chapter II**). Table 4.1 shows the candidate genes selected for this cartilage degradation study. PCR reactions were performed using SYBR® Green PCR master mix and 300nM primer concentration. All data was normalised with a pair of reference genes, HPRT and TBP.

Table 4.1 Candidate gene list of cartilage degradation study

Symbol	Gene name	primers 5'-3'	Efficiency on equine cartilage
MMP-13	Matrixmetalloproteinase 13 (Collagenase 3)	Fw: CTGGAGCTGGGCACCTACTG Rv: ATTTGCCTGAGTCATTATGAACAAGAT	-3.027
MMP-2	Matrixmetalloproteinase 2 (Gelatinase B)	Fw: CCCCGGGCCCTGGAGTTGG Rv: ATCGCTGCGGCCTGTGTCTGTG	-3.358
Col2a1	Type II collagen	Fw: AATAACCTGAATCCAGAAACAACACA Rv: GCGTGACTGGGATTGGAAAG	-3.273
TIMP-1	Tissue inhibitor of metalloproteinase 1	Fw: ATCCCCTGCAAACTGCAGAGT Rv: GCCCTTGTCAGAGCCTGTGA	-3.154
TIMP-2	Tissue inhibitor of metalloproteinase 2	Fw: AGAGTTGTTGAAAGTCGACAAGCA Rv: ACCGAGCGATCACTCAGGAA	-3.098
TIMP-3	Tissue inhibitor of metalloproteinase 3	Fw: CCTACTTCCCCATTAGCCAGTCT Rv: ACAGGGTTTTCTCTGGTTGGTTT	-3.228

Statistical analyses

Two-way ANOVA with Bonferroni post-hoc test was performed using Graphpad Prism software (V. 4.01) to compare the effect of oxygen level and cytokines or the effect of oxygen level for each parameter at each matched time point.

Results

Proteoglycan degradation

The effect of human recombinant cytokines and oxygen tensions on equine cartilage explant (n=3) proteoglycan degradation was measured using the DMMB assay. The accumulative GAG release of non-cytokine treatment explants ranged from 12% at day 1 (Fig 4.1) to 60% of total GAG at Day 28 (Fig 4.2). All cytokine treatments were able to promote GAG loss within the first week of culture (p<0.0001; data not shown). About 25% of total GAG was released into the tissue culture medium from the second day of treatment, and continued to 75% by day 5 (Fig 4.1) and 90% by the end of the long-term experiment (Fig 4.2). However, oxygen tensions had no significant effect on the release of GAG throughout the study (Fig 4.1 and 4.2). There were slight differences between levels of GAG release by control explants between 20% and 1% O_2. In short-term experiments, normoxia treated samples released GAGs more than those under hypoxia while it was opposite in the long-term experiment. However, the difference did not reach statistical significance.

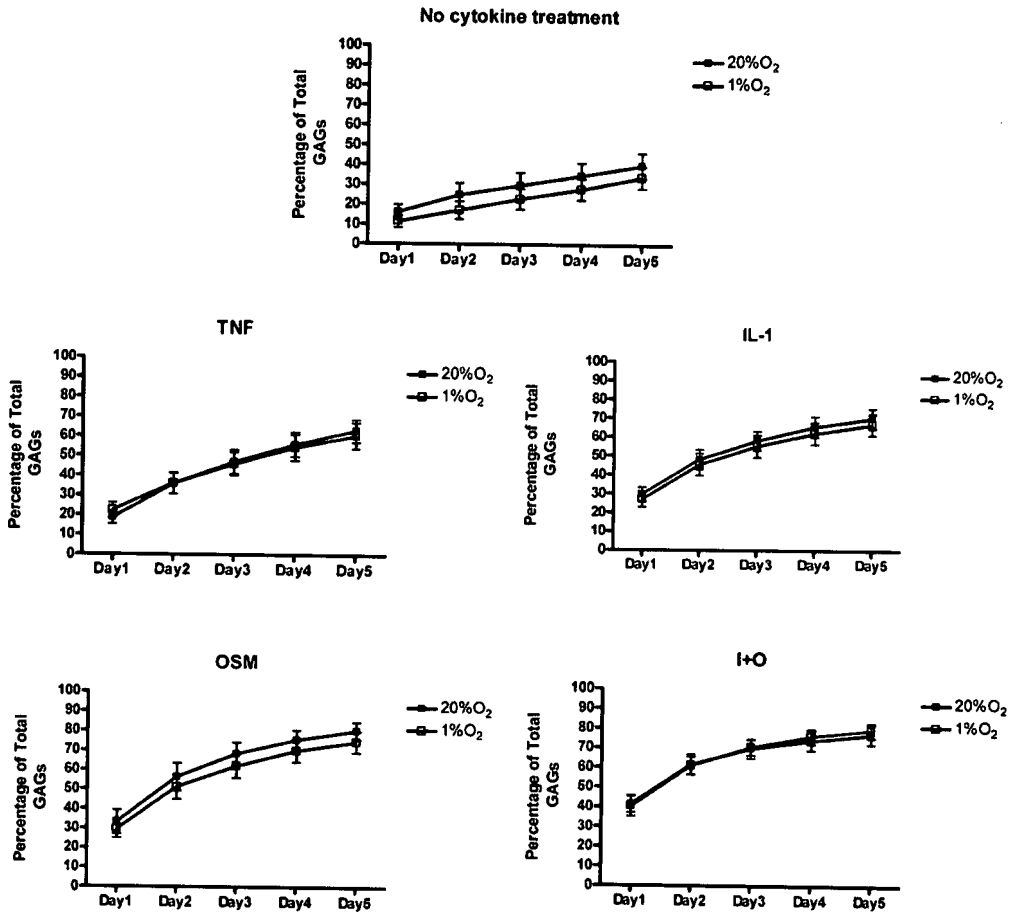

Fig 4.1 Accumulative GAG release during short-term (5 days) treatment with proinflammatory cytokine and hypoxia. The results represent as percentage of total explants GAG content. Equine cartilage explants (n=3) were cultured under 20% or 1% O_2 with 20ng/ml human recombinant IL-1β, TNFα, OSM or combination of IL-1β and OSM (I+O). Each treatment was performed in quadruplicate. Tissue culture medium was collected daily. GAG released into tissue culture medium was analysed by DMMB assay in duplicate manner. The error bars represent SEM.

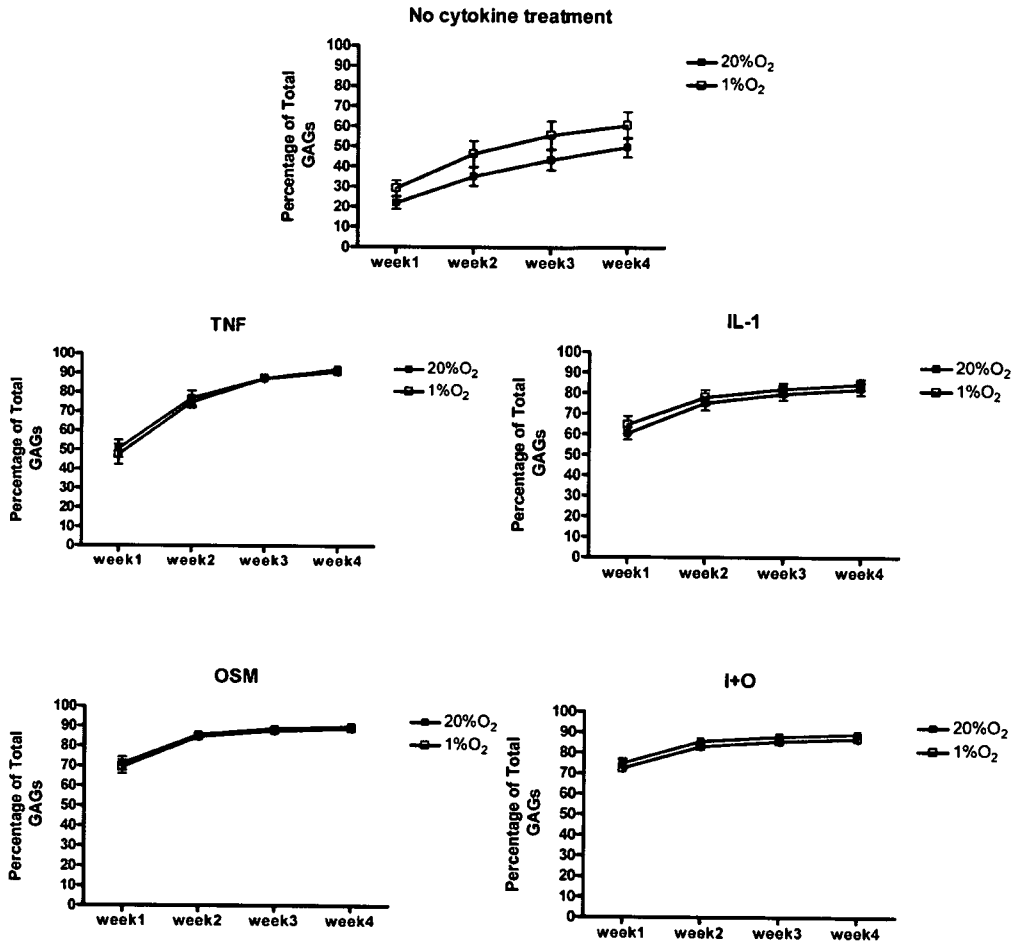

Fig 4.2 Accumulative GAG release during long-term (4 weeks) treatment with proinflammatory cytokine and hypoxia. The results represent as percentage of total explants GAG content. Equine cartilage explants (n=3) were cultured under 20% or 1% O_2 with 20ng/ml human recombinant IL-1β, TNFα, OSM or combination of IL-1β and OSM (I+O). Each treatment was performed in quadruplicate. Tissue culture medium was collected weekly. GAG released into tissue culture medium was analysed by DMMB assay in duplicate manner. The error bars represent SEM.

Collagen release

Collagen degradation was determined using the hydroxyproline assay. Fig 4.3 illustrates the time course of collagen release from equine cartilage explant culture stimulated with various human recombinant cytokines at the two different oxygen tensions. While proteoglycan degradation occured almost immediately, collagen release was undetectable during the first week. Unexpectedly, hydroxyproline in the medium of cartilage treated with TNFα was obviously increased at the second week and continued increasing up to 20% of total hydroxyproline at week 4. This indicates potent TNF-induced collagen degradation whilst other cytokines, including the combined treatment with IL-1 and OSM, had no effect at all. There was no statistical difference in collagen release between normoxic and hypoxic treated samples.

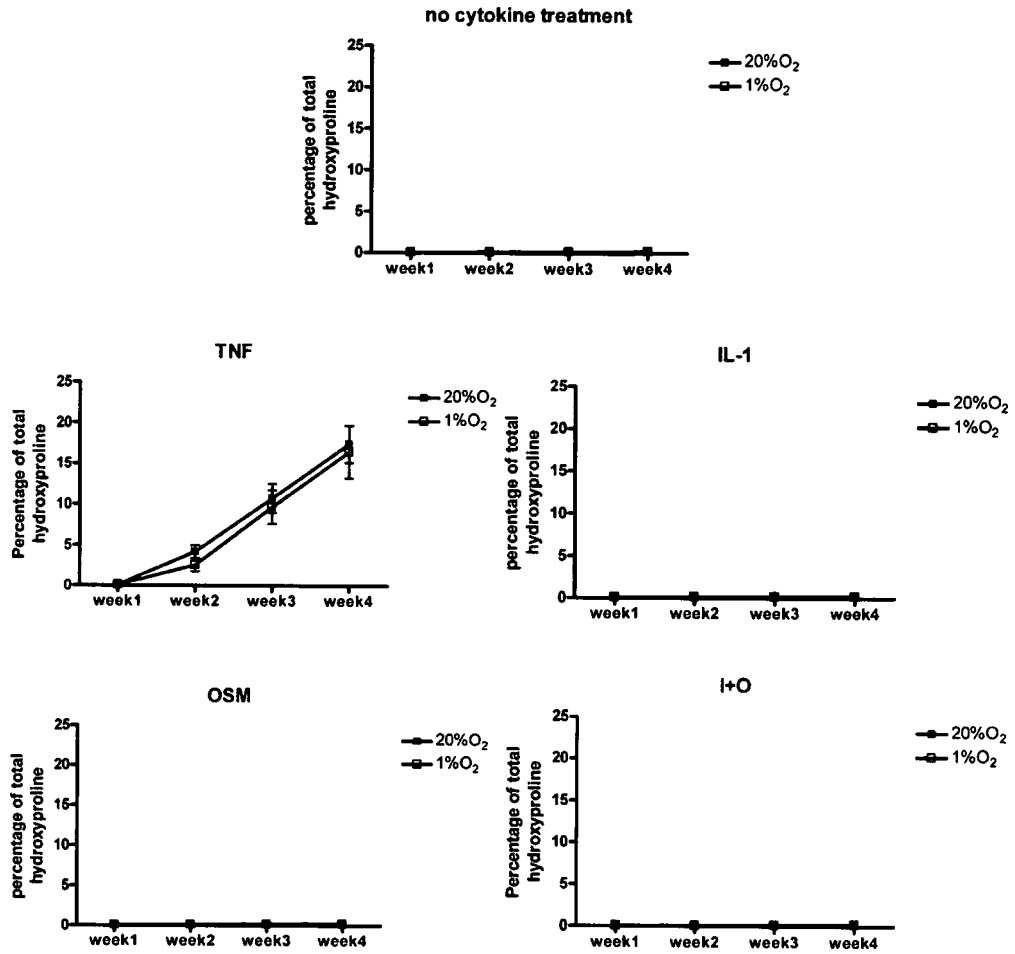

Fig 4.3 Accumulative collagen release during long-term (4 weeks) treatment with proinflammatory cytokine and hypoxia. The results represent as percentage of total explants collagen content. Equine cartilage explants (n=3) were cultured under 20% or 1% O_2 with 20ng/ml human recombinant IL-1β, TNFα, OSM or combination of IL-1β and OSM (I+O). Each treatment was performed in quadruplicate. Tissue culture medium was collected weekly. Collagen degradation released into tissue culture medium was determined by measuring released hydroxyproline (see **Chapter II**). The assay was done in duplicates. The error bars represent SEM.

MMP-13 fluorogenic assay optimization

In testing for interference due to the presence of FCS on the assay, the rate of substrate quenching was sharply increased in the media containing FCS and the fluorescence detection was out of range (data not shown). Therefore, FCS was avoided in this cartilage degradation study.

The two concentrations of the fluorogenic substrate, both 100μM and 10μM were able to give fluorescence within the range that the assay can detect (Fig 4.4) over the 210 minutes recording period. The fluorescence data collected by 180 minutes is shown in Fig 4.5. The MMP-13 activity signals from both concentrations of the substrate generated almost linear curves and the 10μM was selected for the assay.

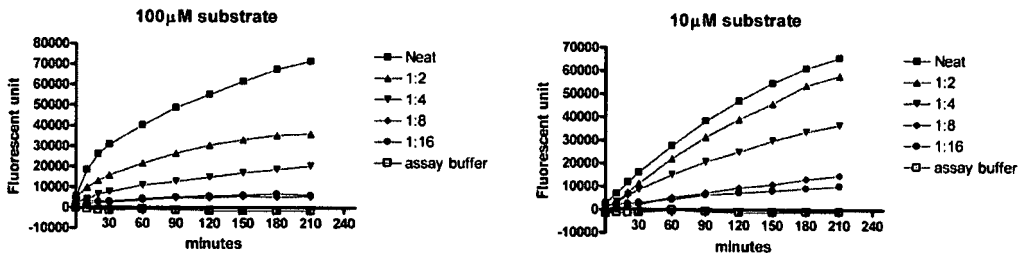

Fig 4.4 Optimisation of the MMP-13 activity fluorogenic assay. Samples were in serial dilutions of medium from LPS-stimulated DH82 cells. Two concentrations, 100μM (left) and 10μM (right), of substrate were used and the fluorescence was observed over 210 minutes of incubation. The backgrounds of the assay were subtracted.

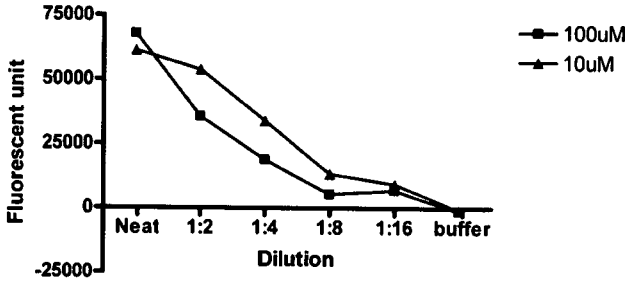

Fig 4.5 Standard curve of MMP-13 activity by fluorogenic assay in serial dilutions of medium from LPS-stimulated DH82 cells with 100μM and 10μM substrate at 180 minute.

MMP-13 synthesis and activation

The activity of MMP-13, a major collagenase of cartilage, was measured in the tissue culture medium harvested from cultures where cartilage explants (n=2) were incubated with and without cytokine treatment at week 3, where the significant collagen degradation in TNF-α stimulated explants was identified. Increases in total MMP-13 were found in response to most cytokine treatments, except OSM. Interestingly, increased levels of active MMP-13 (in the absence of APMA activation) activity were seen in TNF-stimulated explants cultured at 20% O_2 (Fig 4.6). However under 1% O_2, the increased levels of active MMP-13 were not as high as in 20% O_2 (Fig 4.6).

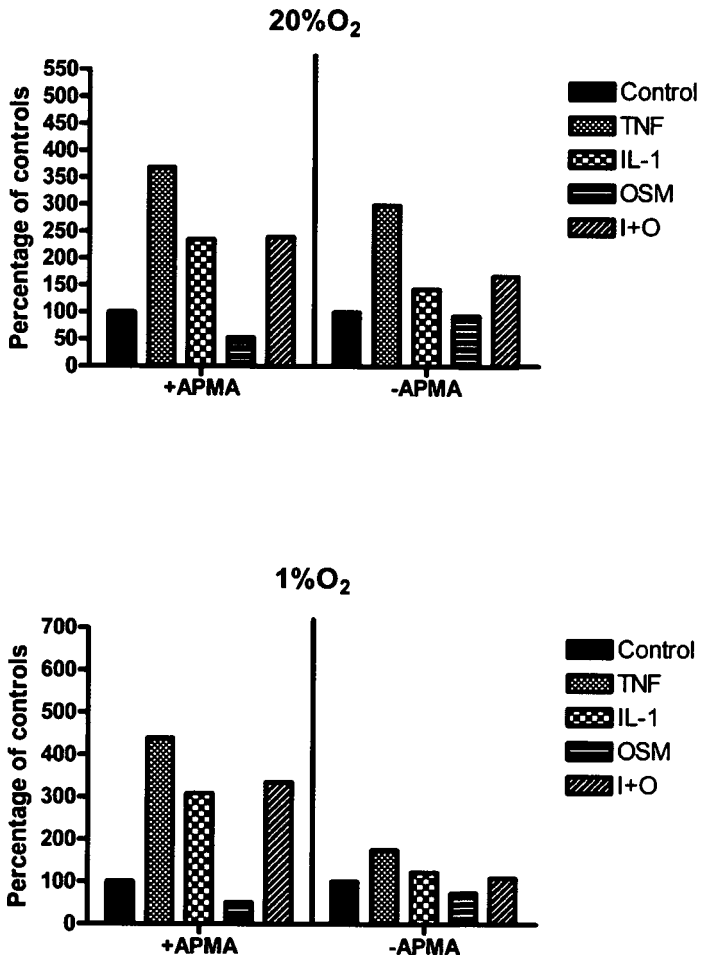

Fig 4.6 MMP-13 activity in tissue culture medium samples of equine cartilage explants with differing oxygen and cytokine treatments. Cartilage explants (n=2 horse) were cultured under 20% or 1% O_2 with 20ng/ml human recombinant IL-1β, TNFα, OSM or combination of IL-1β and OSM at week 3, where the collagen degradation was observed. The enzyme activity was measured by the expression of fluorescence of cleaved fluorogenic MMP-13 substrate. The assay was performed with or without 0.5mM *p*-Aminophenylmercuric acetate (APMA) activation at 37°C for 1 hour in order to detect total or active MMP-13 respectively. The graph shows average fluorescent activity and in each control group, the activity was set as 100 %.

mRNA expression of MMP-13, Col2a1, TIMP-1, TIMP-2, TIMP-3 and MMP-2

MMP-13 mRNA was highly up-regulated ($p<0.001$) in cartilage treated with TNF and IL-1, both in normoxia and hypoxia. OSM had no effect on MMP-13 regulation, while a combination of IL-1 and OSM showed some effect, which was smaller than that of IL-1 alone and reached statistical significance ($p<0.05$) only at 20%O_2 (Fig 4.7). No oxygen effect was found when comparing the expression of each cytokine treatment between normoxia and hypoxia.

While MMP-13 was very cytokine responsive, MMP-2 was constitutively expressed at a very low level and was only significantly regulated ($p<0.05$) by IL-1 in normoxia treatment (Fig 4.7). Type II collagen (col2a1) mRNA was down-regulated ($p<0.05$) by IL-1, OSM and the combined treatment of these two cytokines when cultured under normoxia. However under hypoxic conditions, only OSM significantly inhibited ($p<0.05$) expression of col2a1. Analysis of oxygen effects on MMP-2 and col2a1 expression in individual cytokine treatment did not show any significant differences between hypoxia and normoxia.

TIMP-1 expression was slightly increased by IL-1 and OSM but this was only significant under 1% oxygen treatment (IL-1; $p<0.05$, OSM; $p<0.01$) while combination of these two cytokines significantly increased TIMP-1 transcription in both 20% O_2 ($p<0.05$) and 1% O_2 ($p<0.01$). TNF did not show any effect on TIMP-1 regulation. Although TIMP-1 expression

seemed to be slightly up-regulated by both IL-1 and OSM, TIMP-2 expression was significantly down-regulated by TNF, IL-1 and the combined IL-1-OSM treatments ($p<0.001$). OSM alone also had a down-regulatory effect on TIMP-2 transcription but it was significant only at $1\%O_2$ ($p<0.05$). IL-1 was the only cytokine that played a role in TIMP-3 regulation and even then was only effective in normoxia. The increased TIMP-3 transcription in normoxia-IL-1 treated samples was significantly higher than hypoxic samples ($p<0.05$).

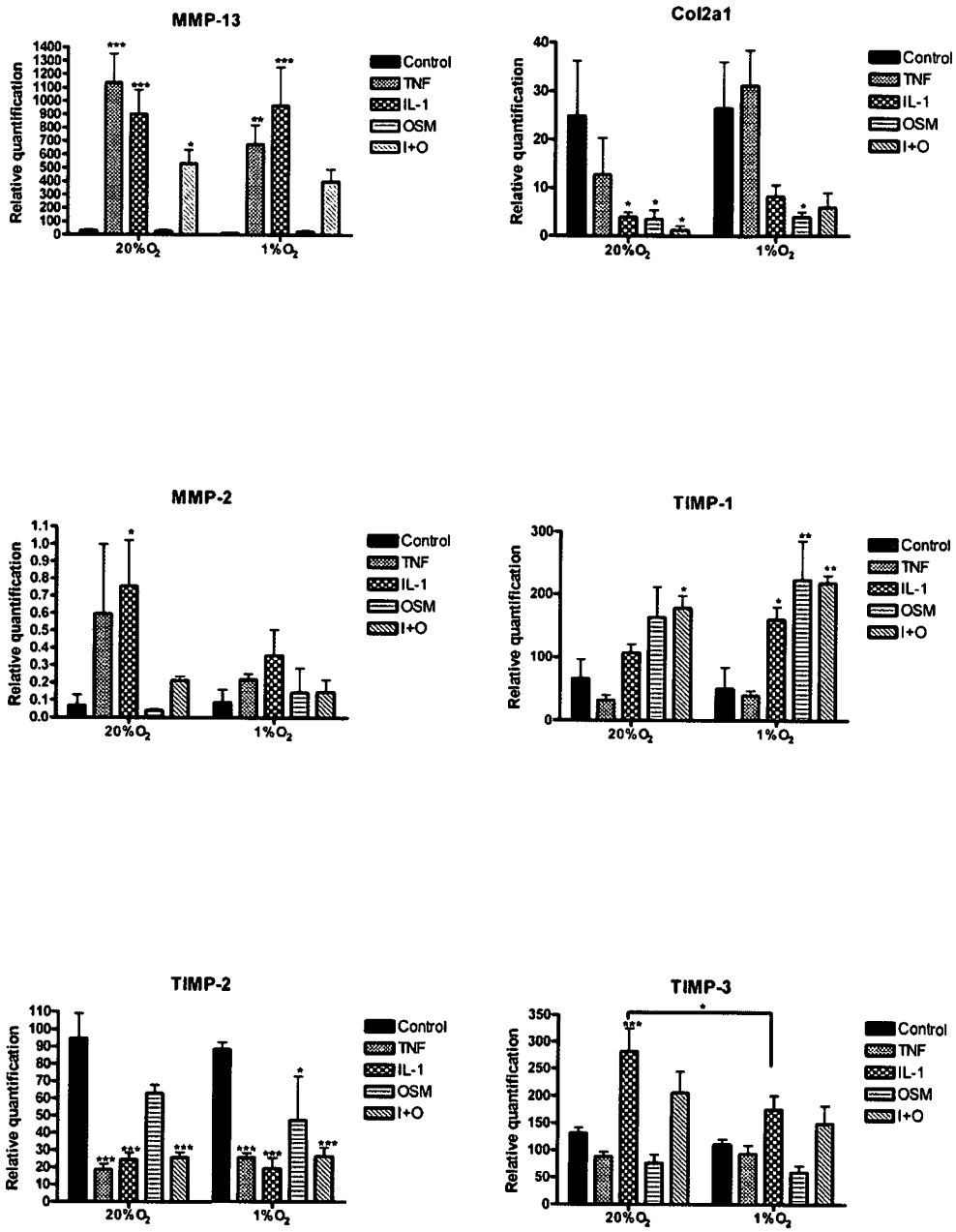

Fig 4.7 Effect of oxygen and proinflammatory cytokines on the expression of genes involved in cartilage turnover (MMP-13, MMP-2, col2a1, TIMP-1, TIMP-2 and TIMP-3). Equine cartilage explants (n=3) were cultured under 20% (normoxia) or 1% O_2 (hypoxia) with 20ng/ml human recombinant IL-1β, TNFα, OSM or combination of IL-1β and OSM for 24 hours. The error bars represent SEM.* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

Hypoxia is believed to be the normal physiological environment of chondrocytes in cartilage, relative to better vascularised tissues. Despite there being numerous publications indicating that a low oxygen level confers an advantage on chondrocyte deposition of cartilage matrix, there is relatively little information about the effects of hypoxia on chondrocyte-mediated catabolic processes.

Our results agreed with other catabolic studies by showing that cartilage responds to cytokines by degrading/releasing proteoglycan very quickly while collagen loss is only detectable some considerable time after GAG loss (Hui et al. 2001; Little et al. 2005; Morgan et al. 2006). In our study the amounts of GAG and collagen released under normoxic and hypoxic conditions were not significantly different, suggesting neither adverse nor positive effects of oxygen level on cartilage structural degradation. However, it is known that aggrecan can be degraded by both aggrecanases and MMPs (reviewed by Dudhia 2005): the question whether there is any change in the proportion of aggrecanse and MMP activities in GAG degradation under different oxygen concentrations still remains.

Stimulation of cartilage explants using IL-1, OSM and the combination of these two cytokines, which is commonly used to induce cartilage degradation in human and bovine studies (Elliott et al. 2001; Hui et al. 2001; Morgan et al. 2006), successfully induced GAG loss but had no

effect at all on collagen release in our equine system. In a study using normal equine cartilage from the metacarpophalangeal and carpal joints, collagenolysis was reported to be inducible by IL-1 alone (Little et al. 2005) but we were unable to replicate this finding using femoral cartilage explants. Surprisingly, TNFα was the only cytokine that was capable of not only inducing GAG release but also collagen degradation and this was reproducible in our system. This is not the first report of a species- or site-specific variation in the response of chondrocytes. In man, collagenolysis by nasal chondrocytes is much easier to stimulate than in articular chondrocytes using the same cytokine stimulations (Morgan et al. 2006). It was suggested that contribution of factors such as cell density, collagen cross links and glycation in each specific site could be the explanation of this variation (Morgan et al. 2006).

Degradation of the collagenous network is due to MMP activity both during normal tissue remodelling and during pathological changes. MMP-13 preferentially cleaves type II collagen (Knäuper et al. 1996 a) and our results showed increased level of total MMP-13 measured at week 3 following TNF and IL-1 treatment of explants. However, approximately 50% of the MMP-13 produced by IL-1 stimulated explants was in the inactive form while TNF-induced explants under normoxia produced mainly (almost 90%) active enzyme. This data correlates well with the

collagen degradation results, confirming the important role for MMP-13 in collagen breakdown.

The extent of collagen release induced in response to TNF treatment was similar in both hypoxia and normoxia and this agreed with the expression of total MMP-13 in both levels of oxygen. However, the active MMP-13 in hypoxia appeared to be lower than demonstrated under normoxia. This observation would suggest that there may be other collagenases (e.g. MMP-1 and MMP-8) or other factors involved in MMP-13 activation under hypoxia. There have been reports indicating that MMP-13 zymogen can be activated by other matrix metalloproteinases such as stromelysins (Barksby et al. 2006 b) and membrane-type1 MMP (MT1-MMP; MMP-14) (Knäuper et al. 1996 b). MMP-14 (MT1-MMP) upregulation has been reported in hypoxic bovine chondrocyte cultures (Martin et al. 2004), therefore MMP-14 could potentially be a factor activating MMP-13 in hypoxic environments.

The response of equine cartilage explants at gene transcription level was also included in our study. As predicted, MMP-13 mRNA expression increased in TNF and IL-1 treatments and showed a significant correlation with the actual enzyme production. Hypoxia was previously reported to enhance the expression of many MMPs including MMP2 and MMP-13 in rabbit fibrocartilagenous chondrocytes (Yamaguchi et al. 2005) but we were unable to identify such an effect in our equine articular cartilage

study. Another study using bovine articular chondrocytes showed that hypoxia did not markedly affect levels of MMP gene expression but significant changes were found following reoxygenation stress (Martin et al. 2004). There was also greater inhibition of type II collagen and aggrecan core protein gene expression when bovine chondrocytes were treated in combination with IL-1 and hypoxia (Martin et al. 2004). Despite inhibition of col2a1 expression by OSM and IL-1 similar to previous report (Kuroki et al. 2005), there was no hypoxia effect on col2a1 gene expression in our study.

The expression of tissue inhibitors of metalloproteinases (TIMP) genes was quite varied and depended on the TIMP species and cytokine stimulation. Our study confirmed findings from previous studies that have identified upregulation of TIMP-1 by many proinflammatory cytokines (Hui et al. 2001; Martin et al. 2004; Kuroki et al. 2005; Morgan et al. 2006); however, one study using bovine nasal chondrocytes identified a down-regulation of TIMP-1 gene expression when stimulated with a combination of IL-1 and OSM (Hui et al. 2001). TIMP-2 expression was reported to increase in an IL-1 stimulated canine chondrocyte study (Kuroki et al. 2005) but it was down-regulated in our study. However, the oxygen level had no effect on either TIMP-1 or TIMP-2 gene expression. TIMP-3 was the only gene whose expression was affected by oxygen but this finding is in contrast to a previous report using chondrocytes from rabbit temporomandibular joints.

While TIMP-3 expression after stimulation with IL-1 was up-regulated and suppressed by hypoxia in our study, Yamaguchi et al.(2005) demonstrated a down-regulation by IL-1 and up-regulation by hypoxia.

Conclusion

In conclusion, among the cytokines used in our study, TNF was able to promote equine articular cartilage matrix degradation and have significant effects along all the degradative pathways studied i.e. GAG release, collagen release, MMP-13 production and activity. Hypoxia seems to have opposite, yet very small effects on cartilage. The response of chondrocytes to cytokines was faster and stronger than to changes in oxygen level resulting in net degradation of cartilage which could not be balanced by low oxygen tension.

Acknowledgement

The author would like to thank Professor Peter Clegg for the qPCR primer sequences used in this study.

Chapter V

EFFECT OF HYPOXIA ON NEO-CARTILAGE SYNTHESIS OF EQUINE CHONDROCYTES

Introduction

Cartilage lacks the ability to completely restore itself once it is injured or degenerated. Attempts have been made to search for a solution to the problem of cartilage repair. One approach, which has been used for human joint repair is cartilage regeneration using autologous chondrocyte implantation (Brittberg et al. 1994; Peterson et al. 2000). More recently, the use of adult stem cells has been explored (reviewed by Chen et al. 2006), with the aim of generating constructs of neo-cartilage with structural and functional properties comparable to native tissue. The induction and stability of chondrocyte phenotype and prevention of chondrocyte dedifferentiation are major concerns in all approaches to cartilage engineering.

Chondrocytes in native tissue synthesise the proteoglycan aggrecan and a heterotypic collagen fibrillar network with type II collagen as major and type IX and XI collagen as minor components, in order to construct and maintain the cartilage matrix (Freeman 1979). The synthesis of these macromolecules has been used to define the chondrocyte phenotype.

Following their isolation from cartilage, expansion of chondrocytes in 2-D monolayer cultures in order to get sufficient numbers of cells drives them towards fibroblastic morphology and synthesis of type I, III and V collagen instead of type II, IX and X (Mayne et al. 1976; Benya et al. 1977; Benya et al. 1978). They also lose the expression of aggrecan core protein (Schnabel et al. 2002). The result of these phenotypic changes is production of an extracellular matrix with inferior biomechanical properties (von der Mark et al. 1977; Watt 1988).

Interactions between chondrocytes and the surrounding matrix are important in the stabilisation of their morphology and functions (Shakibaei et al. 1997). Culture systems which support chondrocytes within three-dimensional environment, thereby maintaining the round appearance of the cells and mimicking their natural habitat, maintain their phenotype stability (Häuselmann et al. 1994; Gregory et al. 1999; Gugala and Gogolewski 2000). Alginate bead (Guo et al. 1989), agarose gel (Benya and Shaffer 1982), polymer scaffolds (Woodfield et al. 2002) and pellet cultures (Zhang et al. 2004) have all been demonstrated to prevent or reverse dedifferentiation of chondrocytes.

Chondrocytes are usually maintained in culture under normoxic conditions. However, oxygen concentrations within cartilage tissue are relatively low compared to other tissues and low compared to most culture environments. Chondrocytes have been reported to adjust their metabolism to compensate

for an environment lacking in oxygen (Rajpurohit et al. 1996) by shifting to anaerobic respiration. Also, evidence of correlation between low oxygen levels and chondrogenesis has been reported e.g. culture of mouse embryo forelimb under low oxygen tension resulted in increased deposition of safranin-o positive matrix (Hirao et al. 2006) and culture of nasal chondrocyte under low oxygen tensions resulted in increased deposition of type II collagen and aggrecan in pellet culture (Malda et al. 2004 a).

Hypoxia has been reported to favour proliferation, matrix synthesis and stimulation of chondrocytic (re)differentiation (see Table 5.1). However, conflicting data have also been produced indicating that findings from studies are not always consistent (see Table 5.1). Several investigators reported that there were no difference in the production of matrix molecules and chondrocyte proliferation between normoxia and hypoxia. Moreover, it was found that those parameters were higher under more aerobic conditions than low oxygen tensions.

Chondrocytes can respond to various cytokines and growth factors by altering their metabolic activity towards anabolism or catabolism. Transforming growth factor-β (TGF-β) has been shown to have a major positive influence on cartilage matrix synthesis (reviewed in Trippel 1995; Grimaud et al. 2002) and has been used in cartilage repair and engineering studies (Glowacki et al. 2005). There is however, no information to date on the

Table 5.1 Summary of data reported in the literature regarding the influence of low oxygen tension on proliferation, cartilaginous matrix synthesis, phenotype stability and (re)differentiation of chondrocytes and other cells (adapted from Henrotin et al. 2005). AC = articular cartilage, NC = nasal cartilage, OA = osteoarthritis, coll = collagen.

Cell or Tissue	Species	Type of culture	Oxygen tensions hypoxia/normoxia	Effect on ECM and cells	Effect on mRNA	Reference
Positive effects of hypoxia						
Primary AC	Bovine	Monolayer	5/20	↑Coll II ↑Coll IX ↓Coll I ↑Proliferation		(Hansen et al. 2001)
Primary AC	Bovine	Alginate	10/20	↑↑Pellet volume (after liberated from bead)		(Grimshaw and Mason 2000)
Primary AC	Bovine	Alginate	5/20	↑Coll II		(Domm et al. 2002)
Primary AC	Equine	Alginate (at 14 days)	5/21	↑Living cells ↓Apoptosis		(Schneider et al. 2004)
Dedifferentiate AC	Bovine	Alginate	5/20		↑Coll II ↑Agg	(Murphy and Sambanis 2001 a)
Dedifferentiate AC	Bovine	Alginate	5/20	↑Coll II ↑Coll IX ↓Coll I		(Domm et al. 2002)
Dedifferentiate AC	Human	Alginate	5/20		↑Coll II ↑Agg ↑SOX9	(Murphy and Polak 2004)
Dedifferentiate NC	Human	Pellet	5/20 1/20	↑GAG ↑Coll II ↑↑GAG ↑Coll II		(Malda et al. 2004 a)
Primary AC	Bovine	Alginate/Collagen I/III membrane culture	5/20	↑Ratio of Coll II/Coll I	↑Ratio of Coll II/Coll I	(Kurz et al. 2004)
Primary AC	Bovine	Porous polyacetic acid construct	5/20	↑GAG		(Saini and Wick 2004)
Primary AC (OA)	Human	Collagen I/III membrane culture	5/20	↑Proline and sulphate corporation		(Scherer et al. 2004)
Dedifferentiate AC	Bovine	Collagen I/III membrane culture	5/20	↑Coll II		(Kurz et al. 2004)
Primary AC	Bovine	Polyurethane scaffold	5/21	↑GAG ↑Coll II ↓Coll I	↑Coll II ↑Agg ↓Coll I	(Wernike et al. 2008)
Dermal fibroblast (for chondroinduction)	Human	Porous collagen sponge containing DBP	5/19	↑C-4-S PG		(Mizuno and Glowacki 2005)
Mice embryo forelimb	Murine	Organ culture	5/20	↑Safranin-O positive area ↓Coll X		(Hirao et al. 2006)

Table 5.1 (Continued)

Cell or Tissue	Species	Type of culture	Oxygen tensions hypoxia/normoxia	Effect on ECM and cells	Effect on mRNA	Reference
Negative effects of hypoxia						
Primary AC	Bovine	Cartilage explant	6/24	↓Sulphate corporation		(Ysart and Mason 1994)
Primary AC	Bovine	Multilayer	5/20	↓GAG ↓Proliferation ↓Collagen		(Murphy and Sambanis 2001 b)
			1/20	↓↓GAG ↓↓Proliferation ↓↓Collagen		
Primary AC	Bovine	Alginate	5/20	↓Pellet volume		(Grimshaw and Mason 2000)
			0.1/20	↓Pellet volume	↓rRNA yield	
Primary AC	Equine	Alginate (at 14 days)	1/21	↓Apoptosis ↑Living cells ↑Necrotic cells		(Schneider et al. 2004)
Pluripotent MS cell line (C3H10T1/2)	Murine	Monolayer	5/20	↓ALP activity ↑GAG ↓Mineralization	↑Coll II ↓Coll X ↓Sox9 ↓Runx2	(Hirao et al. 2006)
No effect of hypoxia						
Dedifferentiate AC	Human	Pellet	5/20		No response in Coll II Coll I SOX9	(Adesida et al. 2007)
Primary AC	Bovine	Porous polyacetic acid construct	5/20	+/-Collagen		(Saini and Wick 2004)

use of TGF-β in combination with hypoxia and it is not known whether or not they have a synergistic effect on matrix synthesis.

This chapter describes studies in which the effects of hypoxia and TGF-β3 on cartilage matrix synthesis were investigated. Studies were carried out using equine chondrocytes in pellet culture system. The study was divided into two experiments. The aim of the first experiment was to compare the effect of 20% and 1% oxygen on cartilage matrix synthesis by equine

chondrocytes. In the second experiment, the matrix production of equine chondrocytes responded to a growth factor, TGF-β3, in combination with low oxygen tension was explored.

Material and methods

Sources of chondrocytes

Chondrocytes used in these studies were from two sources of normal equine cartilage; femoropatellar (stifle; age 5-7 years old) and metacarpophalangeal/ tarsophalangeal (fetlock; age unidentified) joints. Each experiment was performed using cartilage from three different donors. Chondrocytes were isolated and cultured in pellet form as described in **Chapter II**. Sufficient numbers of chondrocytes were isolated from the stifle joints and these were pelleted directly following isolation. However, low yields of chondrocytes were obtained from fetlock joints. Therefore, the fetlock-derived cells, which were used in the second experiment, were expanded as described in **Chapter II** in order to obtain sufficient numbers of cells to culture as pellets.

Chondrocyte pellet culture

Equine chondrocyte pellets were cultured in a 96-well plate system (see **Chapter II**). In experiment 1, the pellets were maintained either under 20%O_2 or 1%O_2 for 14 days. In experiment 2, the pellets were cultured in the same conditions and for the same period of time but with or without the addition of 10ng/ml recombinant human TGF-β3. All treatments were

done in triplicates (two pellets per replicate). Tissue culture media were collected every other day and replenished. At the end of the culture period, chondrocyte pellets were either weighed (only performed in experiment 2 due to limited number of pellets in experiment 1) or processed for RNA extraction using Trizol® or GAG, hydroxyproline and DNA analysis following papain digestion (1mg/ml; 100μl per pellet). Pepsin digestion was used in the pellet preparation for collagen protein extraction (see **Chapter II**).

Analysis of matrix components

Papain digested pellets and tissue culture medium samples were analysed for the deposition and release of GAG and collagen using DMMB and hydroxyproline assay respectively (see **Chapter II**).

Neo-cartilage synthesis gene expressions

Methods of preparing RNA and cDNA preparation were as described in **Chapter II**. However, to improve recovery, the pellets were homogenised using Molecular Grinding Resin before RNA extraction. The expression of aggrecan core protein (agg), type I collagen (col1a1), type II collagen (col2a1) and sox9 was measured using qPCR with SYBR Green® mastermix. Primers were validated as described in **Chapter II** and their sequences are listed in Table 5.2.

Table 5.2 Genes and primers used in cartilage matrix synthesis study

Symbol	Gene name	Primer 5'-3'	Efficiency on equine cartilage
agg	Aggrecan core protein	Fw: GAGGAGCAGGAGTTTGTCAACA Rv: CCCTTCGATGGTCCTGTCAT	-3.322
col1a1	Type I collagen	Fw: AGAGCATTTGCCCAACATTGT Rv: CATGGGAAAGGATGAAAACGA	-3.193
col2a1	Type II collagen	Fw: AATAACCTGAATCCAGAAACAACACA Rv: GCGTGACTGGGATTGGAAAG	-3.273
sox9	sox9	Fw: CTTTGGTTTGTGTTCGTGTTTTGT Rv: AGAGAAAGAAAAAGGGAAAGGTAAGTTT	-3.329

Collagen separation by SDS-PAGE

Collagen molecules were extracted from chondrocyte pellets by pepsin digestion using 100µl of pepsin (1mg/ml in 0.5M acetic acid; Sigma) per pellet for 24 hours at 4°C. Samples were freeze-dried and reconstituted again with H_2O (25µl per pellet). Proteins were denatured by boiling for 4 minutes with sample buffer (see **Chapter II**). β-mercaptoethanol (0.05% (v/v)) was added in order to reduce disulphide bonds in the protein. Equal amounts of each pellet sample was loaded onto each well of SDS gels with a resolving gel of 7% (w/v) acrylamide and a stacking gel of 4% (w/v) acrylamide. Electrophoresis was run for approximately 45 minutes at 200V and subsequently, the gels were stained with Coomassie Brilliant blue stain (see **Chapter II**) for 2 hours. Gels with separated proteins was scanned after destaining (see **Chapter II**) and the image was analysed by Scion Image software (**http://www.scioncorp.com/pages/scion_image_windows.htm**), to compare the intensity of stain associated with protein bands.

Mass Spectrometry

Protein bands separated by SDS-PAGE were dried and sent to the Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, where samples were trypsinised and separated by reverse-phase chromatography and analysed by positive ion electrospray ionization mass spectrometry/mass spectrometry (ESI-MS/MS) using a Quadrupole-Time of Flight (Q-ToF) mass spectrometer to define the amino acid sequences of the peptide fragments (Rousseau et al. 2007). Data acquired were analysed using SWISSPROT and a custom equine database collected from Entrez Protein Database (**http://www.ncbi.nlm.nih.gov**) if available. Otherwise, the alignment between known peptide sequences from other species and equine genome database from Ensembl (**www.ensembl.org**) was performed to obtain predicted equine proteins.

Western blot for type II collagen

Western blots were performed to confirm expression of type II collagen in equine chondrocyte pellet cultures. Pepsin (1mg/ml; 100µl per pellet) digests of each sample were freeze-dried and reconstituted with 25µl H_2O. The reconstituted pellet extracts were mixed with sample buffer (5 times concentration) and denatured with or without reduction by β-mercaptoethanol (0.05%v/v) before loading onto SDS gels with equal amount of each sample. Electrophoresis and western blot on PVDF membrane were performed as described previously (see **Chapter II**).

Primary antibody used in this study was AVT6E3, a monoclonal antibody raised against human type II collagen. The blots were incubated with 1:10 dilution of hybridoma supernatant containing AVT6E3 antibody for overnight at room temperature and with 1:5,000 dilution of alkaline phosphatase conjugated anti-mouse IgG (SIGMA) for 1 hour and room temperature. Interactions with the primary and secondary antibodies were detected by using the alkaline phosphatase substrate, SIGMA FAST^{TM} BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium).

Results

Experiment 1: Study of the effect of hypoxia on pellet cultures using fresh equine chondrocytes harvested from the femoropatella joint (n=3 horses)

1. GAGs and total collagen synthesis

Equine chondrocyte pellets cultured under both 20%O_2 and 1%O_2 were disc-shape and remained intact until harvesting at day 14. Analysis of GAG deposition within the pellet showed that normoxic pellets contained higher (approximately 2 times) amount of GAG than hypoxic pellets (Fig 5.1a). Similarly, accumulative GAG release measured in tissue culture medium from normoxic samples was also higher than hypoxic samples (Fig 5.1c) indicating a suppression of proteoglycan synthesis in chondrocytes cultured under hypoxia. However, the trends of decreasing GAG deposition and release when the pellets were cultured under hypoxia

in this experiment were not significant. There was no difference in collagen deposition and release between the two oxygen levels (Fig 5.1b and 5.1d).

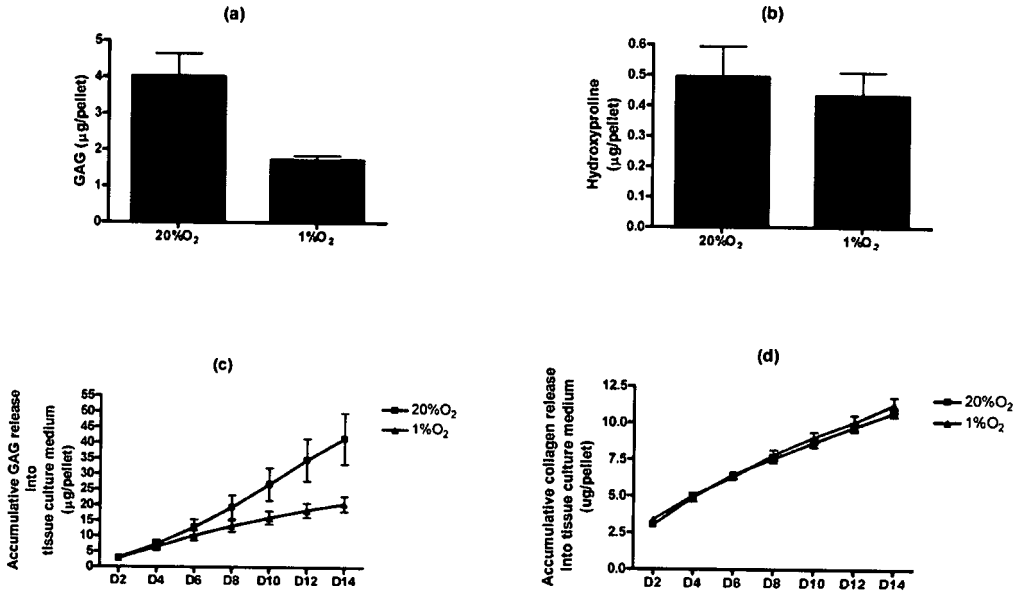

Fig 5.1 Synthesis of GAG and collagen in primary equine chondrocyte pellet cultures (n=3) under 20% O_2 and 1% O_2. (**a**) GAG and (**b**) collagen deposition within pellet cultures Pellets were digested with 1mg/ml papain at 65°C overnight prior to analysis using DMMB and hydroxyproline assays. Tissue culture medium samples were also collected every other day and analysed for GAG (**c**) and collagen (**d**) release. The error bars represent SEM.

2. Identification of collagen type II and other pepsin-resistant components of the pellet matrix by mass spectrometry

To confirm the identity of the bands that were observed by SDS-PAGE, the bands were cut out for mass spectrometry analysis, the result of which are shown in Fig 5.2. Band A (migrating to the same distance as the type II collagen marker, with an apparent molecular weight of 140kDa) and the

lower molecular weight band B were cut out individually. Band C was believed to be the pepsin used for pellet digestion as shown in comigration with the same molecular weight of pepsin. Band C resolved into two bands when the samples were reduced using β-mercaptoethanol; however, they were cut and analysed together.

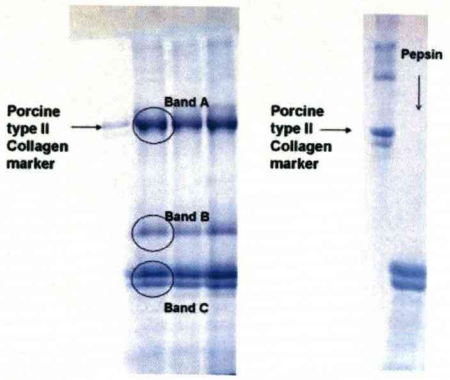

Fig 5.2 Pepsin digested chondrocyte pellets resolved by SDS-PAGE using 7% acrylamide and stained with Coomassie blue. Equine chondrocyte pellets were digested with 2mg/ml hyaluronidase for 2 hours at ambient temperature followed by 1mg/ml pepsin in 0.5M acetic acid (100µl/pellet) at 4°C for overnight. Samples were freeze-dried and reconstituted with H_2O (25µl/pellet) and denatured with reduction by boiling with 14.3M β-mercaptoethanol (Sigma) at 1:20 in sample buffer for 4 minutes. Equal amounts of each pepsin digested pellet were loaded into each well. Electrophoresis of pepsin alone is also shown. A standard preparation of pepsinised porcine type II collagen (2 mg/ml; donated by Dr. Anne Vaughan-Thomas) was used to identify bands co-migrating with type II collagen. Three bands (Band A, Band B and Band C) were analysed by mass-spectrometry analysis.

The peptide fragmentation data from ESI MS/MS analysis was matched against SWISSPROT (http://www.expasy.org/sprot/), and MSDB (M) (http://csc-fserve.hh.med.ic.ac.uk/msdb.html) databases. The data for matches with matrix molecules obtained are summarised in Table 5.3.

Table 5.3 Protein fragments of ESI-MS/MS matched with SWISSPROT and MSDB (M) database.

Sample	Accession number	Protein	Number of peptides matched
Band A	P02458	Collagen alpha 1(II) chain precursor	2
Band B	P12111	Collagen alpha 3(VI) chain	2
Band C	P12111	Collagen alpha 3(VI) chain	1
	P10915	Hyaluronan link protein	1

All of the above related to human sequences. In order to gain more comprehensive analysis, a custom database collected from equine amino acid sequence database from NCBI or predicted sequences of equine collagen proteins by alignment with other species was also included. It was shown that Band A had a matched to alpha 1 chain of equine type II collagen while Band B matched to equine alpha 3 chain of type VI collagen. However, Band C still did not match to pepsin as expected but matched to equine alpha 3 chain of type VI collagen and hyaluronan binding protein (Table 5.4).

Table 5.4 Protein fragments of ESI-MS/MS matched with custom database of equine amino acid sequences. Blue letters represents matched sequences.

Sample	Acession Number	Protein	Sequences
Band A	NP_001075233	α 1 (II)	1011 gptgkqgdrgeagaqgpmgpagpagarglp 1040
Band B	XP_001916467	α 3 (VI)	1961 qraseelrqegvralilvglervanlerlm 1990 2031 pckcsgqrgdrgpigsigpkgvpgedgyrg 2050
Band C	XP_001916467	α 3 (VI)	2031 pckcsgqrgdrgpigsigpkgvpgedgyrg 2050
	Q28381	HA and PG linking Protein	281 kdgaqiakvgqifaawkllgydrcdagwla 310

3. Immunoblotting of pepsin-resistant proteins using a type II collagen specific monoclonal antibody

Western blot was performed using the AVT6E3 antibody to confirm the expression of type II collagen in chondrocyte pellet cultures. Positive immunoreaction was found in porcine type II collagen marker as well as the protein bands migrating to the same distance on the gel (Fig 5.3). The antibody did not cross-react with type I collagen.

Fig 5.3 Type II collagen Western blot. SDS-PAGE (left) and Western blot (right) using AVT6E3 antibody to probe against type II collagen. The standards were porcine pepsinised type I and type II collagen containing samples (donated by Dr. Anne Vaughan-Thomas). Samples were pepsin digested equine chondrocyte pellets. The blot was incubated with supernatant from the 6E3 antibody-producing hybridoma cell line at 1:10 dilution for overnight at room temperature and with alkaline phosphatase conjugated anti-mouse IgG at dilution of 1:5,000 for 1 hour at room temperature.

4. Quantification of type II collagen deposited in the pellet cultures

From SDS-PAGE, ESI-MS/MS data and western blot analysis, it was confirmed that the protein in band A was type II collagen. Coomassie stained gels from pellet cultures (n=2) maintained at 1% and 20% oxygen was scanned and plotted for the intensity of each band by Scion Image software (Fig 5.4).

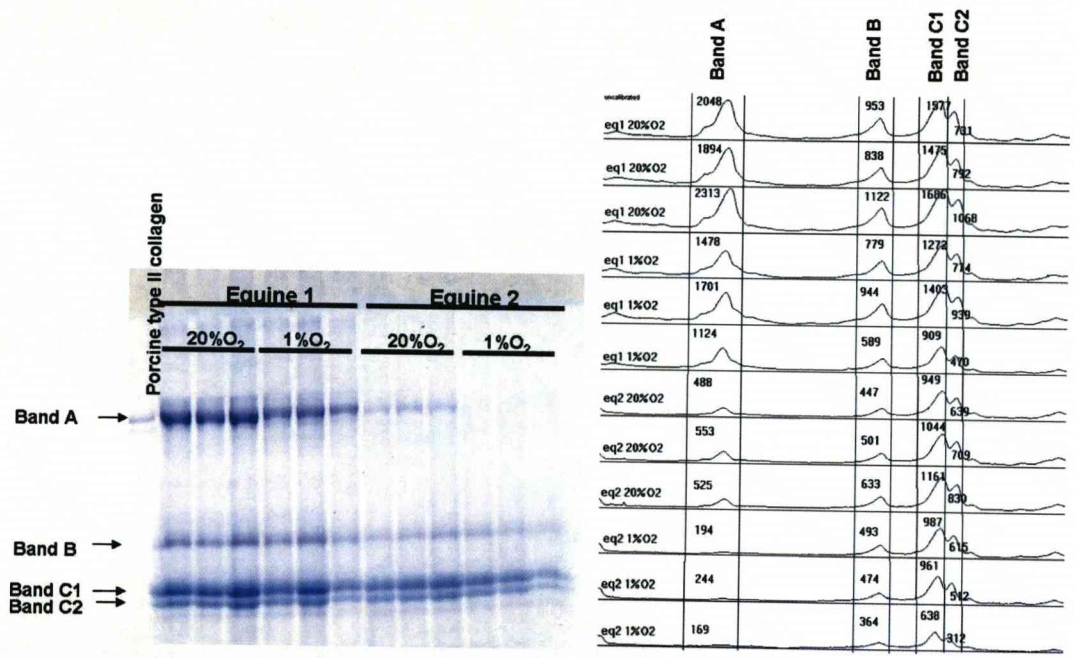

Fig 5.4 SDS-PAGE analysis of pepsin digested chondrocyte pellets (n=2) using Coomassie stain (left) and image analysis for intensity of each protein band by Scion Image (right). Samples were equivalent volumes of pepsin digests of fresh equine chondrocyte pellets loaded onto each well and run alongside a porcine type II collagen marker. Samples were reduced with β-mercaptoethanol prior to electrophoresis.

Intensity values of SDS-PAGE resolved protein bands (Band A, Band B, Band C1 and C2) were compared between hypoxia and normoxia cultures

(n=2). Pellets cultured under hypoxia had lower levels of type II collagen compared to normoxic pellets (Fig 5.5). The intensity of Band B, Band C1 and C2 between the two oxygen levels was also measured but the results showed no trend of higher or lower intensity between the treatments (data not shown).

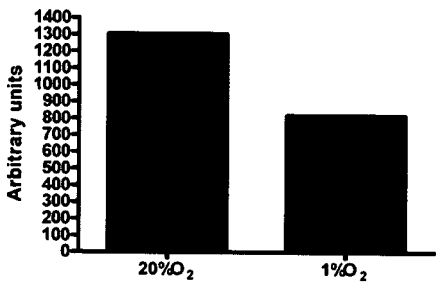

Fig 5.5 Quantification of type II collagen in SDS-PAGE gel. Comparison of average intensity of type II collagen protein bands (Band A) on Coomassie-stained SDS-PAGE gels (after analysis by ScionImage) from pepsin digested pellets (n=2) cultured under normoxia (20% O2) and hypoxia (1% O2).

5. Gene expression studies

Expression levels of genes associated with matrix synthesis was performed using real-timePCR and normalised to expression levels of HPRT and TBP mRNA, which were selected as reference genes (see **Chapter IV**). Aggrecan core protein (Agg), type I collagen (col1a1) and sox9 mRNA were significantly down regulated when the pellets were cultured under hypoxia compared to normoxia (p<0.001, p<0.05 and p<0.01 respectively).

Type II collgen (col2a1) mRNA levels also showed a tendency towards lower expression in hypoxic pellets but this was not significant (Fig 5.6).

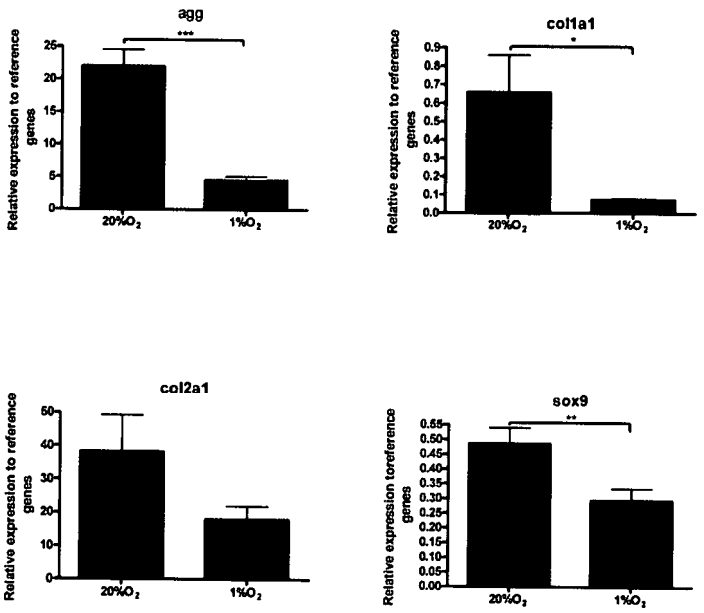

Fig 5.6 Comparison of expression of genes involved cartilage matrix synthesis, type II collagen (col2a1), type I collagen (col1a1), aggrecan core protein (agg) and sox9, in chondrocyte pellets cultured (n=3)under normoxia (20% O_2) and hypoxia (1% O_2). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

***Experiment 2*: Study of the effects of low oxygen tension and TGF-β3 on pellet cultures using first passage equine chondrocytes originally from metacarpophalangeal joint.**

1. Pellet formation, weight and DNA content

Variation in shape and size of pellets was observed (Fig 5.7). Spherical shape was found in normoxic pellets cultured with TGF-β3 supplementation while without growth factor, pellets were disc-like in shape. Under hypoxia (1% O_2), chondrocyte pellets were bigger than under normoxia and were disc-like in shape and hyaline in their appearance. TGF-β3 did not affect the pellet size either in hypoxia or normoxia treatment.

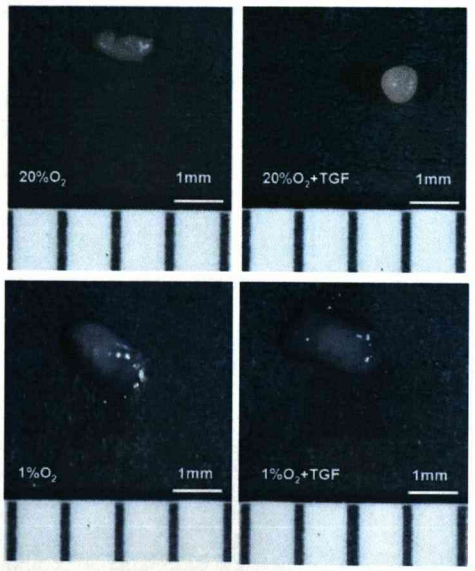

Fig 5.7 Photography of equine chondrocyte pellets. First passage equine (fetlock) chondrocyte pellets were maintained in 20% O_2, 20% O_2 with TGF-β3 (10ng/ml), 1% O_2 and 1% O_2 with TGF-β3. Pellets were photographed immediately upon removal from culture medium at 14 days. Scale bar =1mm.

Pellet weight followed the trend of pellet size (Fig 5.8a). There was no TGF-β3 effect on pellet weight but weight of hypoxic pellets were significantly increased compared to normoxic ones (p<0.001). Also pellets treated by hypoxia alone were significantly heavier than those treated by TGF-β3 alone. Differences in DNA content in each treatment were not significant although 1%O_2 pellets seemed to contain more DNA per pellet than 20%O_2 pellets (Fig 5.8b).

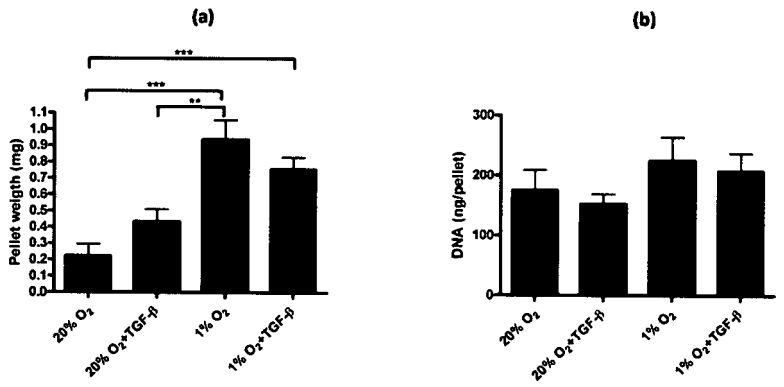

Fig 5.8 Comparison of weight and DNA content of first passage equine chondrocyte pellets (n=3). (a) weight and (b) DNA content of cultures maintained under normoxia (20%O_2), normoxia with TGF-β3 (10ng/ml), hypoxia (1%O_2) and hypoxia with TGF-β3 (10ng/ml). The error bars represent SEM. ** p<0.01; *** p<0.001.

2. GAG and total collagen synthesis

GAG and collagen content of pellets and medium were analysed by DMMB and hydroxyproline assays, respectively. In this experiment, only minimal differences between oxygen levels and TGF-β3 treatments were observed (Fig 5.9). For collagen synthesis, similar to the previous experiment, we observed no difference. GAG synthesis was not inhibited

by hypoxia but seemed to be highest when the pellets were cultured in hypoxia without TGF-β3 treatment although this was not significant.

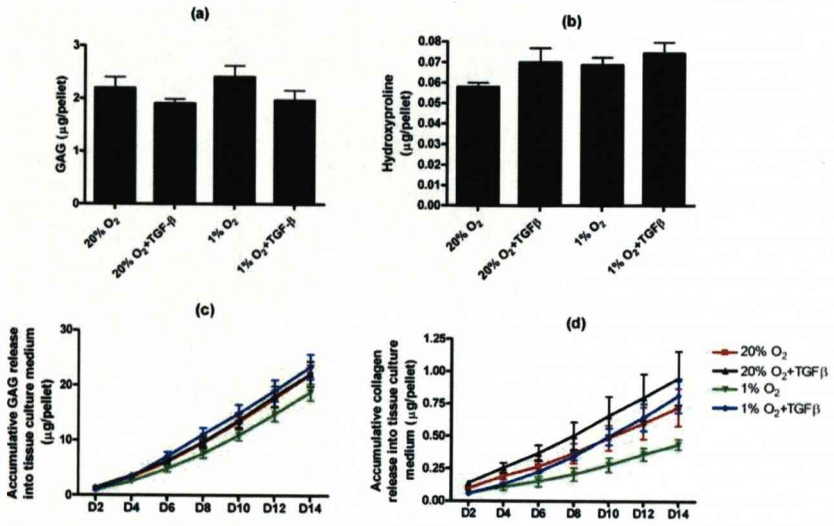

Fig 5.9 Deposition and release of GAG and collagen of first passage equine chondrocyte pellet cultures (n=3). (**a**) GAG and (**b**) collagen deposition in comparing of culures maintained normoxia and hypoxia and with or without TGF-β3 supplement. Accumulative GAG and collagen release into tissue culture medium are also shown (**c** and **d**, respectively).

3. Quantification of collagen type II in pellet matrix by SDS-PAGE and staining with Coomassie blue

Coomassie stained proteins resolved by SDS-PAGE of pepsin digested first passage equine chondrocyte pellets (n=3) were analysed using Scion Image software (Fig 5.10). Variation between animals was observed however, pellets cultured under hypoxia appeared to produce more type II collagen (Band A) than normoxia although this difference was not significant. TGF-β3 had no significant effect on collagen type II synthesis (Fig 5.11). Band B proteins in this study were not apparent. The levels of

the bottom two bands (Band C1 and C2) were also measured (Fig 5.10) but were not altered in response to treatments (data not shown).

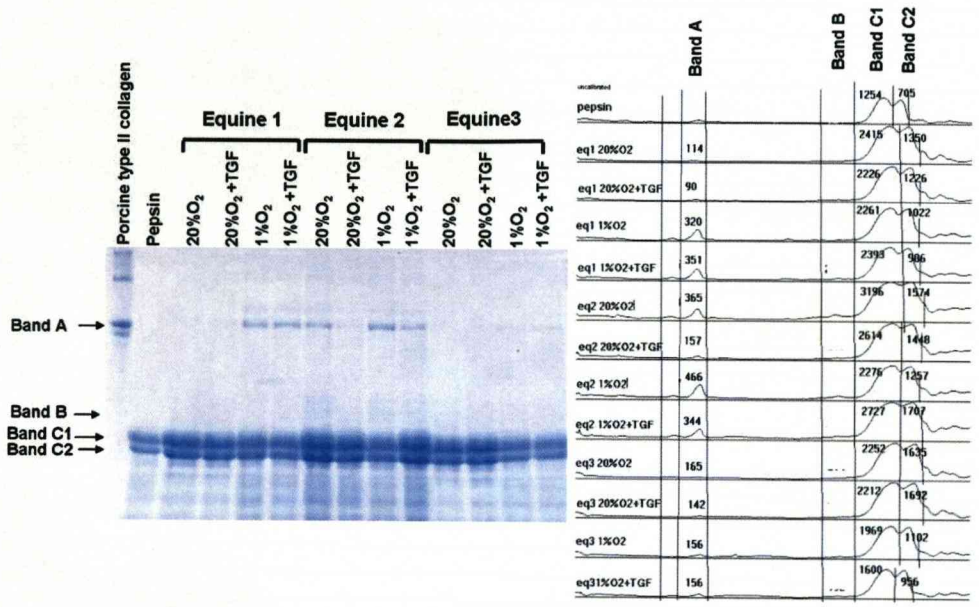

Fig 5.10 Pepsin digested pellet (n=3) protein separation on SDS-PAGE with Coomassie stain (left) and image analysis for intensity of each protein band by Scion Image (right). Samples loaded were equivalent volumes of pepsin digests of first passage equine chondrocyte pellets run alongside the porcine type II collagen marker. Samples were reduced with β-mercaptoethanol prior to electrophoresis.

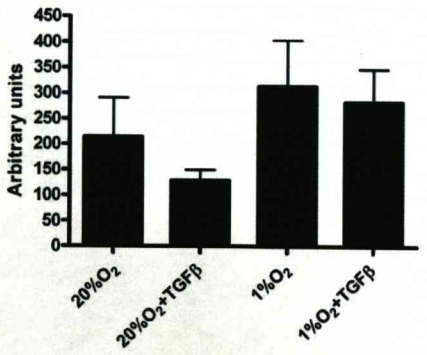

Fig 5.11 Comparison of intensity of type II collagen protein bands (Band A) on Coomassie stained SDS-PAGE gel (after analysis by ScionImage) from pepsin digested pellets (n=3) cultured under normoxia (20%O_2), normoxia with TGF-β3 (10ng/ml), hypoxia (1%O_2) and hypoxia with TGF-β3. The error bars represent SEM.

4. Gene expression studies

Analysis of gene expression levels in the first passage equine chondrocyte pellets was performed by real-time PCR is shown in Fig 5.12. Surprisingly, the results were in contrast to the previous experiment. Expression of aggrecan core protein (agg) mRNA was not suppressed but was highest when the pellets were under hypoxia without TGF-β3 treatment and this correlated well with result of GAG deposition using DMMB assay. However, the significant difference on agg mRNA expression appeared only between pellets that were treated with TGF-β3 alone and those that were treated with hypoxia alone. Hypoxia showed the down-regulation effect on col1a1 gene. TGF-β3 seemed to up-regulate col1a1 but this was not significant. There was no significance in type II collagen (col2a1) mRNA expression. Interestingly, it was clearly shown that hypoxia significantly up-regulated Sox9 expression, however, no TGF-β3 effect was found on this gene.

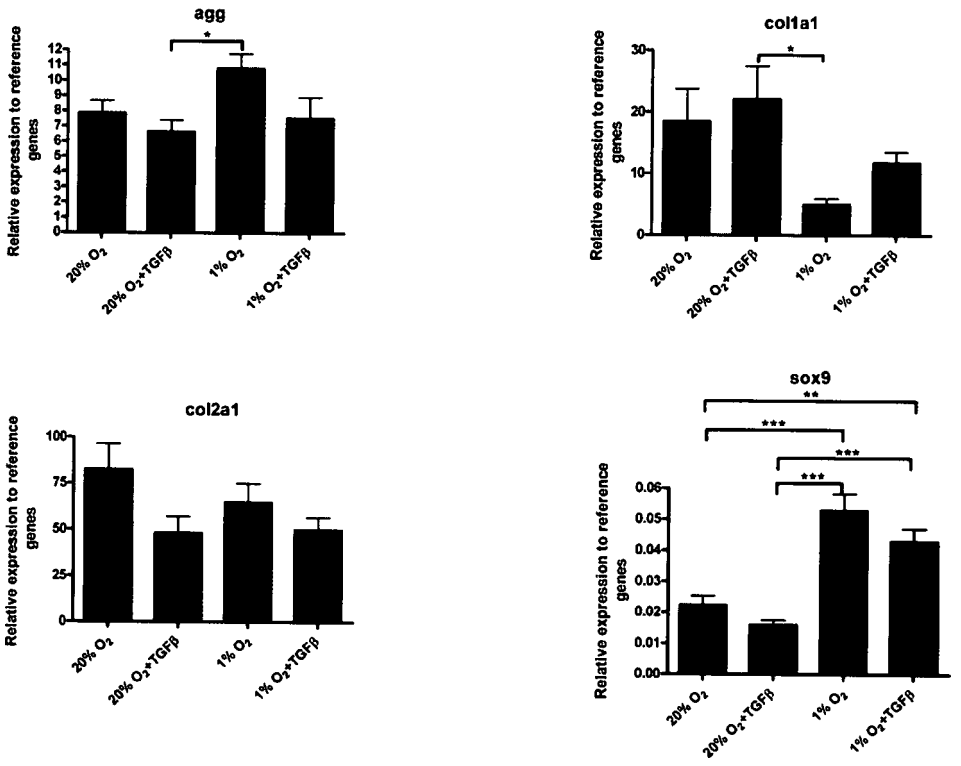

Fig 5.12 Comparison of expression of genes involved in cartilage matrix synthesis, aggrecan core protein (agg), type II collagen (col2a1), type I collagen (col1a1) and sox9, in first passage equine chondrocyte pellet (n=3) cultured under normoxia (20%O_2), normoxia with TGF-β3 (10ng/ml), hypoxia (1%O_2) and hypoxia with TGF-β3. The error bars represent SEM.* p<0.05; ** p<0.01; *** p<0.001.

Discussion

Pellet culture was selected for use in this study as it was demonstrated that it stabilises chondrocyte phenotype and the matrix deposited is similar to native cartilage (Zhang et al. 2004). Oxygen is one important factor in cartilage tissue engineering due to the fact that cartilage, *in vivo*, is relatively hypoxic. Although chondrocytes are able to survive under a wide

range of oxygen tensions (Brighton et al. 1974; O'Driscoll et al. 1997; Grimshaw and Mason 2000; Schneider et al. 2004), atmospheric condition has been considered to be hyperoxic to chondrocytes. There is evidence of adverse effects of high oxygen tension on chondrocyte metabolism i.e. senescent changes (Nevo et al. 1988) and apoptosis (Schneider et al. 2004) possibly resulting from disturbance of oxidation-reduction status of the cells (Lotz et al. 1999). In contrast, low oxygen levels that mimic oxygen tensions within native cartilage have been reported to have beneficial effects on chondrocyte survival, phenotype stability and (re)differentiation; however, findings of hypoxic effects are controversial (see Table 5.1).

Our results also showed conflicting data. Hypoxia had no positive but rather negative effects on neo-cartilage synthesis in one experiment while it was able to induce matrix production in another experiment. By using pellet cultures of fresh equine chondrocytes isolated from femoro-patella joints, hypoxia appeared to inhibit GAG and type II collagen synthesis. This also correlated well with gene expression of agg, col2a1 and sox9. On the other hand, opposite findings were shown in another experiment using pellet cultures of first passage equine chondrocytes originally from metacarpo/tarsophalangeal joints. In the latter study, hypoxia increased not only pellet size and weight but also sox9 expression regardless of TGF treatment.

It has been reported that chondrocytes in each zone of cartilage have different matrix production activities (Aydelotte et al. 1992). It is therefore possible that chondrocytes from mixed zones may cause variation in their response to stimuli including oxygen levels. Although, in both experiments described in this chapter, chondrocytes were collected from full-thickness cartilage, the two joints differ in articular cartilage thickness [average thickness stifle; 1.5-2 mm (Frisbie et al. 2006) and fetlock; 0.5-1 mm (Norrdin et al. 1999)] and may therefore contain different proportions of cells from each zone which could contribute to the differing results observed.

Another possibility which may equally affect chondrocyte responses is the passage of cells in monolayer prior to pellet culture. Previous reports have demonstrated the ability to regain the chondrocytic phenotype of cells previously dedifferentiated in monolayer culture before redifferentiation process is impaired (Murphy and Polak 2004; Darling and Athanasiou 2005). It is possible that our equine chondrocytes were dedifferentiated during expansion stage and may have been unable to fully recover their phenotype to that of primary chondrocytes during the subsequent redifferentiation phase of pellet culture.

In general, hypoxia seemed to have greater effects, either positive or negative, on GAG synthesis than on total collagen production as measured by the hydroxyproline assay. However, the hydroxyproline assay

determines total collagen synthesized by the pellets but is not specific to any types of collagen. To be more specific, analysis of type II collagen was performed by measuring the intensity of Coomassie stained protein bands on SDS-PAGE gels loaded with pellet protein extracted with pepsin. Pepsin cleaves the majority of non-triple helical collagenous proteins, including the non-collagenous termini (telopeptides) of collagen molecules. Hence, it releases and solubilises the triple-helical domains of collagen from the highly cross-linked telopeptide regions. Type II collagen bands were confirmed by comparison with a standard on SDS-PAGE, by mass-spectrometry and also, western blot. Image analysis of SDS-PAGE resolved protein revealed that although oxygen tension had not much effect on total collagens, type II collagen was responsive to hypoxia (again either positive or negative depending on the experimental design).

As the amount of total collagen was not disturbed by hypoxia, the question of what type of collagen compensated for the alteration of type II collagen deposition remains. There is a possibility that chondrocytes produced type I collagen to compensate for type II but this may only apply to the second experiment that showed a lower level of type II collagen but higher collagen type I gene expression under normoxia; this cannot explain chondrocyte behaviour in the first experiment. It should be noted that the alpha 1 chains of both type I and type II collagen co-migrate on SDS-PAGE, but no alpha 2 chain of type I collagen was detected, nor was type I

collagen detected by mass spectrometry in the analyses. Perhaps more than one type of collagen should be considered when trying to account for the difference in collagen composition. Type VI collagen was identified by mass-spectrometry results but this type of collagen is a very large molecule (McDevitt et al. 1988) and unlikely to migrate as far as the position of the band that was indicated to be type VI collagen unless it has been degraded. However, the expression of type VI collagen should be one of the priorities for future cartilage synthesis study as it is abundant in pericellular matrix, which promoted neo-synthesis of cartilage matrix (Graff et al. 2003). It would also be interesting to investigate other types of collagen e.g. type IX and XI, which are involved in stabilization of type II collagen.

Our chondrocyte pellets were very small (~1mm in diameter) compared to previous reports. Zhang and co-workers (Zhang et al. 2004) demonstrated that chondrocyte pellets from sterna of chick embryos were approximately ten times larger in size (~9-10 mm in diameter). However, taking into account the reduced numbers of cells (20-fold less cells) used in the experiment described in this chapter, the pellet formation was actually comparable to theirs. However, difference in species, age or developmental stage of the tissue and pellet culture system should be considered.

In the study of interaction between TGF and hypoxia, we found that normoxia reduced pellet size and weight regardless of TGF treatment and that these two parameters correlated well with each other but were not in

complete agreement with GAG content, which rather followed the trend of DNA content. The reduction of GAG content of normoxic culture was proportionately much less than the extent of reduction in pellet weight. It is known that weight of cartilage tissue is mainly from water trapped by GAG, however, difference in collagen fibre orientation between pellet culture and native cartilage (Zhang et al. 2004) also probably leads to difference in expansion capacity of the matrix. It was also possible that normoxia may alter fibre orientation within the pellets so that it does not allow ECM expansion while absorbing water despite GAG existing. The shape and appearance of normoxic pellets also support this speculation. Again, differences in fibrillar collagen may indicate altered collagen type.

TGF has been shown to regulate chondrocyte matrix synthesis (Glowacki et al. 2005) and a study has demonstrated an additive effect between a growth factor (FGF-2) and hypoxia on chondrogenic signals in meniscus cells (Adesida et al. 2006). So we hypothesised that we could find an interaction between TGF and hypoxia on chondrocytes. However, TGF had no significant effect on matrix synthesis in our study either under normoxia or hypoxia. A review by Trippel (Trippel 1995) has suggested that the response of chondrocytes to TGF is complex. The actions of TGF are varied and sometime opposite depending upon surrounding conditions. A study using bovine nasal cartilage found an increasing GAG turnover but not deposition after TGF treatment (Xu et al. 1996) while we observed

no increase but rather decrease in both proteoglycan synthesis and release. However, for type II collagen deposition, both our and the previous studies showed similar findings that TGF had no effect. These support the finding that TGF effects can be unpredictable when the types of cells and/or culture conditions are changed. In addition, a study using mesenchymal stem cells indicated that TGF alone was not sufficient to induce chondrogenesis but was able to synergise with Sox-9, -5 and -6 proteins (Sox-trio) (Ikeda et al. 2004). The use of Sox-trio proteins in combination with TGF should be for a subject for further study as it is probably able to provide more promising results than TGF alone. In addition, It was shown that GAG synthesis in chondrocytes expanded in monolayer in the presence of a growth factor prior to seeding on scaffolds was increased (Martin et al. 1999; Martin et al. 2001). Therefore, alternatively, applying TGF treatment at the expansion phase of chondrocytes before pelleting should be considered for future study.

Finally, of course, we should consider the possibility that the use of recombinant human growth factors to stimulate cells from other species may not be reproducible. However, until recombinant equine growth factors become available for comparison, this question remains unanswered.

Conclusion

The finding from our study in equine chondrocytes indicated that the effects of hypoxia on cartilage matrix neo-synthesis are generally equivocal. Oxygen tensions may promote cartilage matrix synthesis when chondrocytes have previously been dedifferentiated but inhibit such function in primary cells. TGF had effect neither on its own nor in combination with hypoxia. Hence, it is probably not the best chondroinductive factor for equine neo-cartilage synthesis. Chondrocyte behavior on matrix synthesis under different oxygen levels may seem complicated due to involvement of many factors. However, a number of considerations for further studies of the effects of oxygen levels include microscopic evaluation of the orientation of ECM substances, synthesis of other molecules related to ECM network e.g. type IX and XI collagens, decorin, biglycan etc., the effects of chondrocyte passage (from the same joint) and also interactions with other factors e.g. IGF or FGF. Our current data are interesting and suggest that exploring the contribution of these additional analyses in future studies would provide a more complete picture and help us to understand the role of hypoxia on cartilage matrix production and formation.

Acknowledgements

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Manchester, for mass-spectrometry analysis and Professor Peter Clegg for the qPCR primer sequences used in this study.

Chapter VI

HIF-1ALPHA PROTEIN EXPRESSION IN EQUINE CHONDROCYTES (PRELIMINARY STUDY)

Introduction

Hypoxia inducible factor (HIF) is a key regulator of cellular responses to hypoxia. Chondrocytes can modulate their oxygen consumption in response to hypoxia (Haselgrove et al. 1993) and remain viable even after prolonged culture in <0.1% O_2 (Grimshaw and Mason 2000). It is possible that HIF may also play important roles in chondrocyte survival and maintenance of fundamental homeostasis in the normally hypoxic articular cartilage and may also be involved in maintaining chondrocytes in pathologic cartilage (Yudoh et al. 2005).

HIF-1α is a short lived protein and the kinetics of HIF-1α expression varies among different organs. In liver and kidney, as shown by immunoblot analysis, HIF-1α reaches maximal levels after 1 hour and gradually decreases to baseline levels after 4 hours of continuous hypoxia (Stroka et al. 2001). In the brain, however, HIF-1α is maximally expressed after 5 hours and declines to basal levels by 12 hours (Stroka et al. 2001). By immunohistochemistry, the HIF-1α subunit was not detectable in normal human articular cartilage or benign cartilage tumours (Lin et al. 2004).

However, chondrosarcoma cell lines and chondrocytes maintained under 0.2% O_2 expressed this α subunit after as little as 30 minutes of hypoxia and expression continued for at least 48 hours (Aigner et al. 2004).

HIF expression has been widely studied in tumours and cancer cells (Masson et al. 2001). Regulation of HIF-1 in many cancer cells is manipulated at the transcription level by cytokines and hormones (Déry et al. 2005) as well as some effects being observed at the post-translation level. However, this is not the case in the physiology of normal cells, including chondrocytes, in which HIF is mainly regulated post-translationally. Therefore, HIF studies in normal cells tend to focus on protein levels using antibodies, i.e. immunoblot and immunohistochemistry, to identify HIF protein expression. Also the electrophoresis mobility gel shift assay has been used to detect HIF DNA-binding activity after the protein is identified (Garner and Revzin 1981).

So far, there has been no report of HIF protein expression in the horse. To study how equine chondrocytes function under hypoxia, HIF was potentially the first important key leading us to a better understanding of any physiological adaptation. This part of the study aimed to identify and compare the expression of HIF-1α in equine chondrocytes in hypoxic and normoxic conditions. Human cell lines, prostate cancer (PC3) and chondrosarcoma (C28), were used as positive controls of HIF-1α expression. PC3 have been reported to express this protein (Palayoor et al.

2003) whereas there is no report for C28 but they are very similar to normal human chondrocytes in other regards. Although this initial part of the hypoxia study was not able to deliver clear answers due to the limitations of the detection techniques, there was some interesting information generated which may be useful for further HIF studies.

Materials and Methods

Reagents

Anti-human HIF-1α polyclonal antibody was from SantaCruz (SC-10790). Anti-human HIF-1α monoclonal antibody was from BD transduction Laboratories (H72320-050). Sepharose 4B CNBr powder was from Pharmacia Fine Chemicals. Chondroitinase ABC (EC 4.2.2.4) was from SIGMA (C-3667). Trypsin EDTA was from Invitrogen (25300-054). For Western blotting, protein standards were SeeBlue® pre-stained standards from Invitrogen (LC-5925) and High weight range molecular markers from Sigma (M3788).

Tissue sources and cell cultures

Equine cartilage (aged 4-8 years) and muscle samples were derived from horses which were euthanased for clinical reasons. Isolated chondrocytes were cultured either in a 2-dimensional system as monolayers or in a 3-dimensional system as alginate bead cultures. Canine chondrocytes (from normal joints of dogs euthanased for clinical reason apart from joint diseases with the owner consent) in alginate bead cultures were provided

by Dr. Sue Bell. A human prostate cancer cell line (PC3) and a human chondrosarcoma cell line (C28) were cultured and used as positive controls for HIF-1α protein expression. The cell lines were cultured only in the monolayer system because of their aggressive proliferation.

For hypoxia experiments, cells were cultured under 2-5% oxygen tension, which has been used to provide hypoxic conditions for chondrocyte cultures in many experiments (Grimshaw and Mason 2000; Hansen et al. 2001; Domm et al. 2002; Richardson et al. 2003; Barry and Murphy 2004; Matsushita et al. 2004; Moussavi-Harami et al. 2004; Schneider et al. 2004), or were cultured under 20% oxygen for normoxia control. A seven-day period was selected as the basic duration for all chondrocyte treatments, while the cell lines were cultured under such conditions no longer than 3 days to prevent their overgrowth. The viability of cells was checked in each culture system by the trypan blue dye exclusion method.

Nuclear extraction from equine muscle

All steps were performed at 2-8°C. Horse muscle (total 2 g) was diced into small pieces and each 0.5g tissue aliquot was mixed with 2.5ml low salt buffer (see **Chapter II**: nuclear extraction). The tissue was homogenised using a Dounce homogeniser. The muscle suspension was then centrifuged at 850g for 10 minutes at 4°C. After discarding the first supernatant, homogenisation with low salt buffer was repeated and the suspension was then centrifuged again at 15,000g for 3 minutes at 4°C to separate the

cytosolic fraction. After removed the cytosolic fraction, the sediment was re-suspended and homogenised with high salt buffer (see **Chapter II**: nuclear extraction) and agitated on ice for 2 hours before it was centrifuged at 15,000g for 5 minutes at 4°C. The supernatant, which contained the nuclear extract was collected and measured for protein content by Lowry's protein assay (see **Chapter II**).

Immunoblotting

Whole cell lysates or nuclear proteins (see **Chapter II**) were extracted from alginate bead or monolayer cultures. In some experiments, samples were pre-treated with enzymes prior to SDS-PAGE. Chondroitinase ABC was applied in an experiment to test whether or not proteoglycan (produced by chondrocytes in alginate bead culture) interfered with protein resolving in PAGE gels. Cell lysate samples were treated with the enzyme (0.0075 units per sample) and incubated at 37°C for 4 hours before sample buffer was added. Trypsin EDTA treatment was used in an experiment to eliminate remaining pericellular proteins of chondrocytes, after they had been liberated from alginate beads, which may interfere with protein migration on SDS-PAGE. Chondrocytes were pre-treated with or without trypsin EDTA (1:10 dilution in PBS), which was eventually neutralised by DMEM supplemented with 10%FCS, before the cells were washed with PBS and processed for nuclear extraction.

Unless stated otherwise, for PAGE, 20μg of protein were loaded into each well of 7% vertical SDS-PAGE gels. SeeBlue® pre-stained standards (Invitrogen) were used as molecular weight markers and blot transferring controls on each blot while the Sigma high molecular weight range was used as dual standards in some experiments. DTT (5mg/ml) was added to the sample buffer in order to reduce disulphide bonds in the protein samples. The gels were run at 200V for electrophoresis and the resolved proteins were transferred to PVDF (Millipore) membrane using the Tris-Glycine buffer system at 150 V for 1 hour. Membranes were blocked by 5% skimmed milk. Primary antibodies were HIF-1α polyclonal antibody (SantaCruz) or HIF-1α monoclonal antibody (BD transduction) (see **Chapter II** for antibody dilutions and secondary antibodies used in this study). The blots were developed by enhanced chemiluminescence (ECL) (see **Chapter II**).

Stripping and reprobing immunoblot

Immunoblots that had been developed using non-precipitating substrates such as ECL can be stripped and reprobed if the blots are stored securely in PBS at 4°C (for long-term storage, a preservative such as 0.02%sodium azide may be considered). Thus, after immunodetection with one antibody, the blots were submerged in stripping buffer (100mM 2-mercaptoethanol, 2%SDS, 62.5mM Tris-HCl pH 6.8) and incubated at 50°C for 30 minutes with gentle agitation. The membrane was washed two times each for 10

minutes with PBST. Probing new antibodies were then used as previously described.

HIF-1α separation by affinity chromatography

To purify putative HIF-1α protein detected in immunoblots, affinity chromatography was performed. Samples were prepared from 2 sources; 7.5μg and 175μg of nuclear extracts and cytosolic fractions respectively of equine chondrocytes in alginate beads (approximately 1 million cells) and 1.2mg and 6mg of nuclear extracts and cytosolic proteins respectively from equine muscle (2g). Samples were diluted in PBS to make up to 4 ml each.

Sepharose 4B CNBr powder was transformed to a semi-solid gel by adding an excess of 0.1M $NaHCO_3$, pH 9.0. After leaving the gel to swell properly, 160μg of anti-HIF-1α polyclonal antibody (SantaCruz) was applied for coupling with 5 ml of gel at room temperature overnight on a roller mixer. Non-ligated CNBr sites on the Sepharose gel were blocked by 50mM glycine in 0.1M $NaHCO_3$, pH 9.0 for 2 hours at room temperature. 0.1M sodium formate pH 3.0 and 5mM $NaHCO_3$ were respectively used for washing the gel over a sintered glass funnel under suction. After that, the Sepharose gel was transferred into the 10 ml syringe cylinder which was connected to a pump and rinsed with PBS at 140 drops (~1ml) per fraction. Each subsequent equine samples were then loaded to the system separately and incubated on the gel matrix for 30 minutes at room temperature. Non-binding proteins were run through the column with

excess PBS. The bound proteins were eluted with 5 mM NaOH pH 11.2 and neutralized with 0.7M TrisHCl pH 7.8 at 1:10 dilution. The eluted fractions were collected and measured by a spectrophotometer at 280 nm wavelength. Pooled fractions containing each peak of protein were measured by a spectrophotometer (for protein content) and were run on 7% SDS-PAGE gels and transferred to PVDF membrane prior to probing with anti-HIF-1α polyclonal antibody (SantaCruz) in an attempt to identify purified or enriched HIF-1α.

Results

Immunoblot

The PC3 cell line has been previously shown to express HIF-1α (Goda, 2003). Therefore, these cells were chosen to be a positive control. However, our ECL immunoblot using anti-HIF-1α polyclonal antibody (SantaCruz) results showed positive immunoreactive bands migrating at approximately 55-64 kDa instead of 120 kDa as identified in previous reports. The chondrosarcoma cell line (C28) also showed positive immonoreaction at 55 kDa but to a much lesser extent. In contrast, equine chondrocytes in alginate beads cultures showed intense but diffuse bands migrating at approximately 148-250 kDa (Fig 6.1). Equine chondrocytes in monolayer cultures also expressed 55-60 kDa proteins reactive with HIF-1α antibody similar to C28 and PC3 (Fig 6.2). Different oxygen level treatments (20% and 2%O_2 or 20% and 5%O_2) did not have a significant

effect on HIF-1α immunoblot results (Fig 6.1 and 6.2). Using DTT to reduce the disulphide bonds in samples did not change the appearance of immunoreactive bands compared to unreduced samples (Fig 6.3). Positive reactions of around 90-120 kDa protein were found in samples from canine chondrocytes in alginate bead cultures (Fig 6.4). Sample treated with trypsin EDTA in this experiment did not show different results compared to untreated samples. There was no non-specific reaction from the secondary antibody in any cell culture samples. However, unexpectedly, non-specific reactions of HIF-1α polyclonal antibody (SantaCruz) were detected with Sigma standards (Fig 6.4).

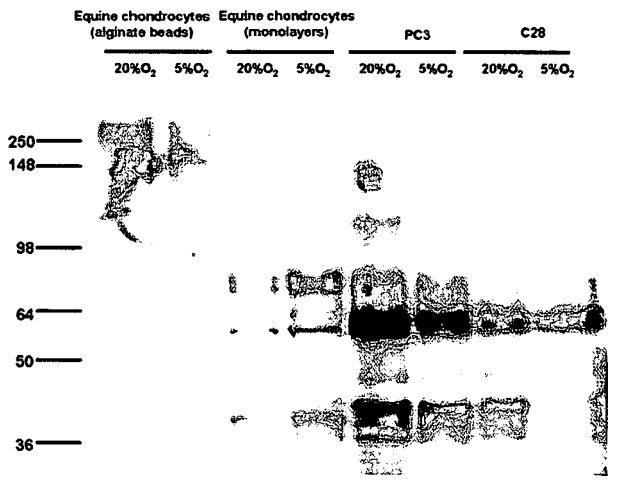

Fig 6.1 Western blot chemiluminescence; immunoreactivity of anti-HIF-1α with nuclear extracts (20μg; unreduced) from samples cultured under 20% or 5% O_2. Samples were equine chondrocytes in alginate bead and monolayer cultures, prostate cancer cell line (PC3) and human chondrosarcoma cell line (C28) maintained either under 20% or 5% O_2 for 1 week for equine chondrocytes and for 3 days for PC3 and C28 cell lines.

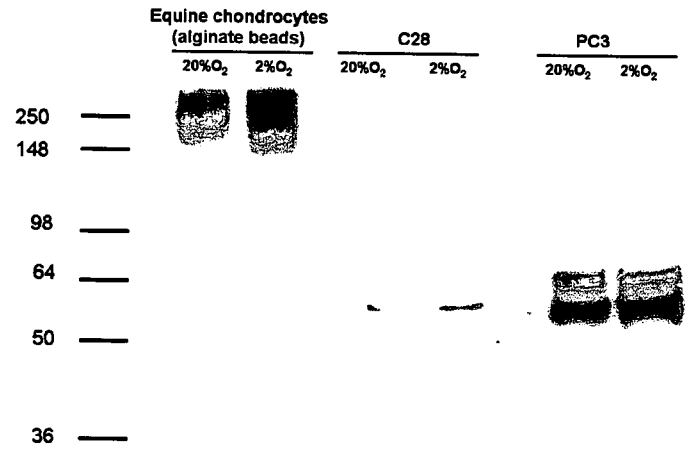

Fig 6.2 Western blot chemiluminescence; immunoreactivity of anti-HIF-1α with whole cell lysates (20μg; unreduced) from samples cultured under 20% O_2 or 2% O_2. Sample were equine chondrocytes in alginate bead, prostate cancer cell line (PC3) and human chondrosarcoma cell line (C28) maintained under 20% O_2 or 2% O_2 for 1 week for equine chondrocytes in alginate beads and 3 days for PC3 and C28 cell lines.

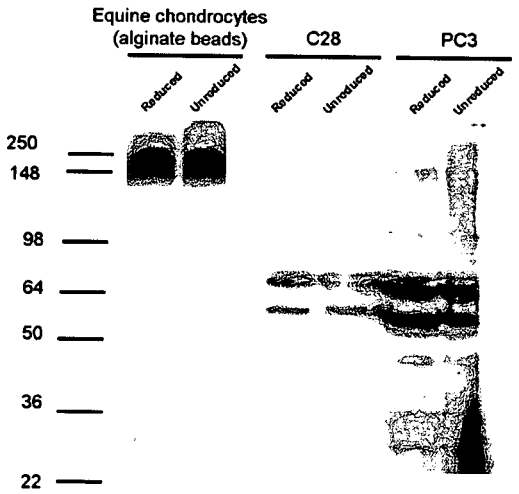

Fig 6.3 Western blot chemiluminescence; immunoreactivity of anti-HIF-1α with whole cell lysates (20μg) with or without disulfide reduction. Samples were equine chondrocytes in alginate bead culture, C28 and PC3 maintained at 2% O_2 for 1 week for equine chondrocytes and for 3 days for C28 and PC3 cell lines. Reduced samples were treated with 5mg/ml DTT and boiled for 4 minutes before loading on the SDS-PAGE.

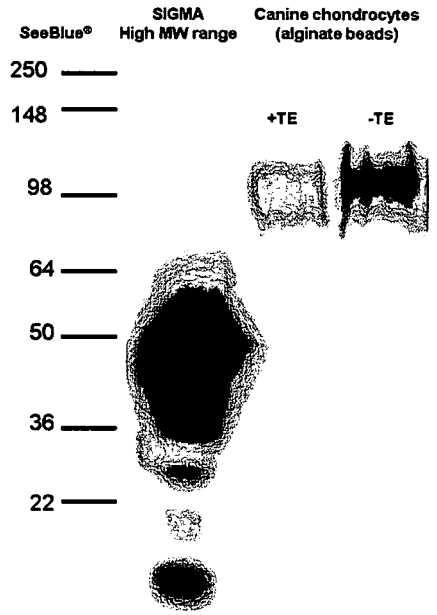

Fig 6.4 Western blot chemiluminescence; immunoreactivity of anti-HIF-1α with nuclear extract (20μg; unreduced) of canine chondrocytes in alginate beads (provided by Dr. Sue Bell) previously treated with or without 1:10 trypsin EDTA (Invitrogen) in sterile PBS after being liberated from alginate beads. Trypsin was neutralised by DMEM supplemented with 10% FCS before the cells were washed with PBS and processed for nuclear extraction. Non-specific binding of HIF-1α polyclonal antibody to the Sigma molecular weight marker (high weight range) is also shown.

It was possible that these very high molecular protein bands which consistenly appeared in alginate bead samples resulted from protein migration blockage by proteoglycans, which would have been produced during the 3-dimensional culture. To eliminate proteoglycans, samples from alginate beads were treated by chondroitinase ABC at 37°C for 4 hours before loading on to the electrophoresis gels. However, the result remained similar between chondroitinase-treated and untreated samples (Fig 6.5).

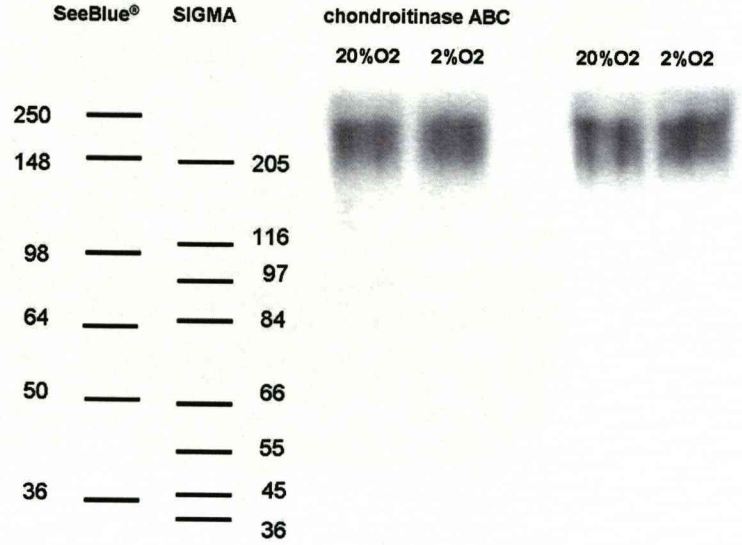

Fig 6.5 Western blot chemiluminescence; immunoreactivity of anti-HIF-1α polyclonal antibody with whole cell lysates of equine chondrocytes in alginate beads with or without chondroitinase ABC treatment. Cultures were maintained under 20% O_2 or 2% O_2 for 1 week. The whole cell lysates (20μg) were incubated with a 1:10 dilution of chondroitinase ABC in PBS at 37°C for 4 hours before loading on SDS-PAGE with sample buffer.

Anti-HIF-1α antibody (BD transduction) was applied as an alternative antibody which might potentially give a more specific reaction with HIF-1α protein than the polyclonal antibody. An immunoblot, previously probed with polyclonal antibody, was stripped (No remaining immunoreaction was found by ECL after stripping) and reprobed with monoclonal antibody. The binding reaction of the monoclonal antibody differed from that obtained previously with the polyclonal antibody. Alginate bead samples had no reaction with this antibody at all while chondrocyte monolayers and the C28 cell line expressed bands at around

55 kDa. However, the antibody gave non-specific reaction with samples of the PC3 cell line (Fig 6.6).

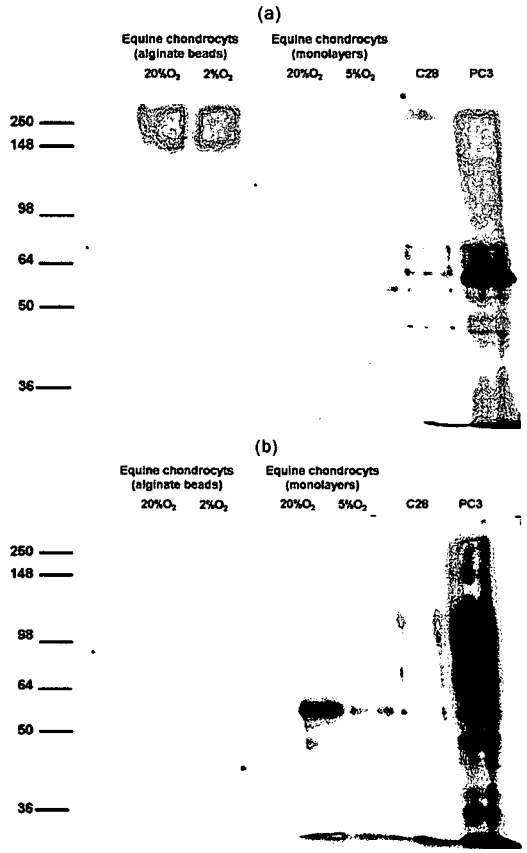

Fig 6.6 Western blot chemiluminescence; comparing immunoreactivity of anti-HIF-1α polyclonal antibody (1:200 dilution; SantaCruz) and anti-HIF-1α monoclonal antibody (1:250 dilution; BD transduction). Whole cell lysates (20μg; unreduced) were prepared from equine chondrocyte in alginate bead and mononalyers maintained either in 20% O_2, 5% O_2 or 2% O_2, PC3 and C28 cells were cultured in 5% O_2. **(a)** immunoreaction of anti-HIF-1α polyclonal antibody. The blot was stripped and re-developed with ECL to ensure complete elimination of the remaining activity after stripping. The blot was reprobed with anti-HIF-1α monoclonal antibody. The result of monoclonal antibody is shown in **(b)**.

HIF-1α purification

Affinity chromatography was used in an attempt to purify or concentrate putative HIF-1α protein identified in immunoblots. Unfortunately, beginning with very small amounts of nuclear protein prepared from chondrocyte cultures resulted in fractions too dilute to detect any peaks of protein (data not shown).

Potential sources of equine HIF-1α positive control were then considered. Although there has been no report of HIF-1α in the horse, positive HIF-1α expression in mouse muscle (Stroka et al. 2001) suggested that horse muscle may be useful source of equine HIF-1α. Therefore, horse muscle was collected and prepared for nuclear proteins. Affinity chromatography using anti-HIF-1α polyclonal antibody was performed. The pooled fractions from each peak were analyzed by western blot chemiluminescence. Unfortunately, there was non-specific immunoreactivity of the anti-rabbit IgG, which was used as the secondary antibody, with horse muscle extracts (Fig 6.7).

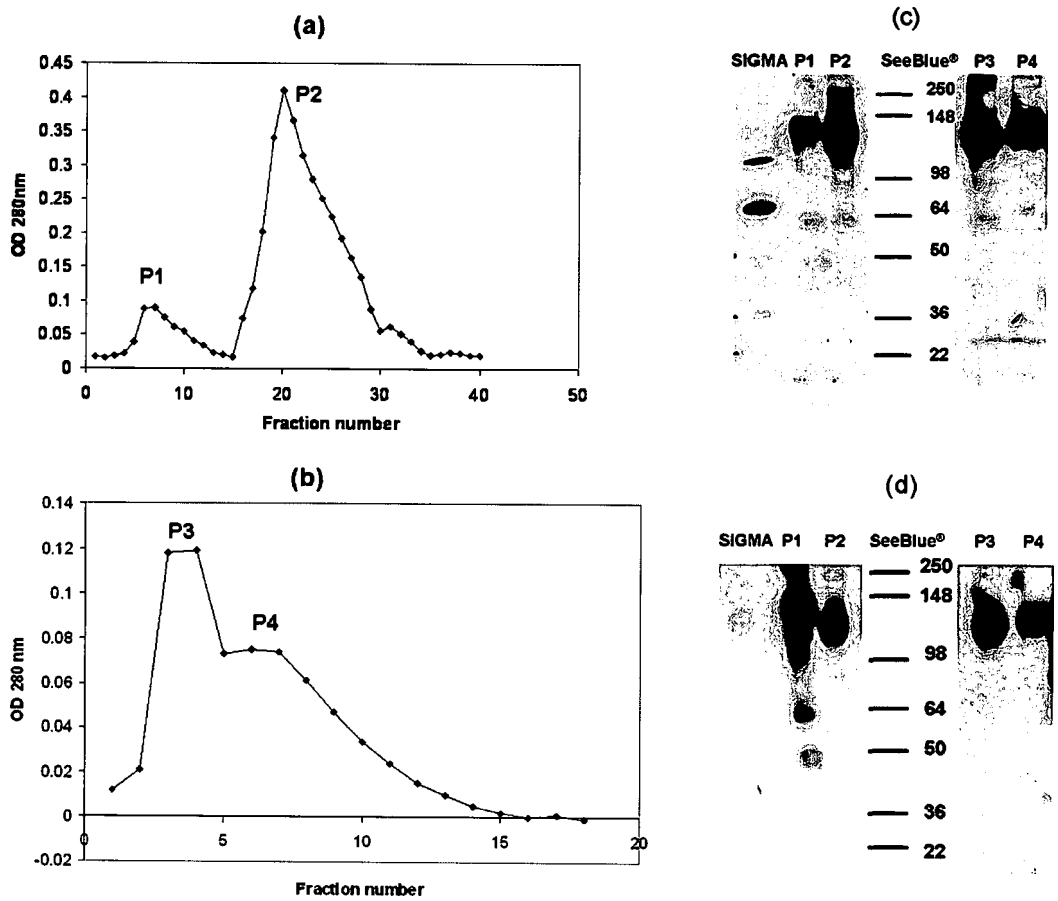

Fig 6.7 HIF-1α affinity chromatography of nuclear (**a**) and cytosolic (**b**) protein fractions from equine muscle extracts using anti-HIF-1α polyclonal antibody. Samples from pooled fractions of each peak (nuclear extract : P1 = fraction 4-10; P2 = fraction 15-30, cytosolic fraction: P3 = fraction 3-4; P4 = fraction 6-7) of chromatography were run on SDS-PAGE along with SIGMA (high molecular weight range) and SeeBlue® markers and followed with ECL immunoblot (**c**). Secondary antibody control is also shown (**d**).

Discussion

Using anti-human HIF-1α polyclonal antibody, canine chondrocytes in alginate beads were the only cell type in our study that expressed protein bands close to expected molecular weight (~120 kDa) for HIF-1α. Equine

chondrocytes in alginate bead cultures consistently showed much larger proteins detected on immunoblots while monolayer cultures of equine chondrocytes and PC3 and C28 cell lines showed protein bands of much lower molecular weight. Apart from these variations in molecular weight, we also observed no oxygen regulation of the proteins that had positive reaction with this antibody. Absence of non-specific reaction from the secondary antibody indicated that the reactions were entirely from this HIF-1α polyclonal antibody.

A study by Chun and his colleagues (2002) identified a human HIF-1α splice variant protein, HIF-1α^{516} (60-75 kDa) which was not regulated by oxygen levels (Chun et al. 2002). Although alginate bead cultures were unlikely to express this HIF-1α form because the protein bands were much higher in molecular weight, it was possible that our monolayer cultures expressed this splice variant of HIF-1α. If so, this may indicate preferential expression of the lower molecular weight variant by cells cultured in 2-dimension (monolayer) system.

However, to date, there is no report of any HIF-1α isoform that is larger than 120 kDa. We thought that the very high molecular weight protein expressed by equine chondrocytes in alginate beads was possibly from binding between HIF protein and proteoglycan or pericellular proteins produced by 3-D cultured chondrocytes but this was ruled out following

digestion using chondroitinase (for PG) and trypsin EDTA (for pericellular protein).

Affinity chromatography using the HIF-1 polyclonal antibody with equine muscle nuclear proteins was able to detect 120 kDa bands. Unfortunately, there was, to some degree, non-specific binding from the secondary antibody with these extracted muscle proteins. If the HIF-1α antibody had reacted with HIF-1α in equine muscle extracts, it could have confirmed that HIF-1α with molecular mass of 120kDa was not expressed by equine chondrocytes. However, the non-specific reaction of the antibodies limited the ability to confirm HIF-1α expression in horse muscle.

It is also possible that the protein bands detected in our immunoblots were not HIF-1α. The 120 kDa HIF-1α may have been expressed in equine chondrocytes in undetectable level or was not expressed at all. There are several reasons why HIF-1α may be absent in our experiments. First, the seven-day duration of hypoxic treatments in chondrocytes may be long enough to drive chondrocytes back to a basal stage as they are in cartilage where HIF-1α may be undetectable (Lin et al. 2004). Second, HIF-1α is a short lived protein. A study demonstrated *in vitro* that nuclear HIF-1α protein level was rapidly decreased within 4-8 minutes upon reoxygenation (Groulx and Lee 2002). In our experiment, reoxygenation conditions were unavoidable, especially during harvesting procedures which took more than 5 minutes, unless the fully hypoxic system was used. Third, it is also

possible that the chondrocytes may preferentially express other α-subunits such as HIF-2α, which was reported to play a dominant role in hypoxic chondrocyte gene regulation studies (Lafont et al. 2007), than HIF-1α. If these statements are true in our experiments, the results could be indicating just the non-specific reactions from HIF-1α antibodies. It is unfortunate that the two anti-human HIF-1α antibodies we had available were unable to detect this molecule in the horse. Maybe there are significant species differences in HIF-1α protein structure or the levels of expression are much lower in the horse.

Conclusion

HIF-1α has been considered to be a key factor regulating cellular homeostasis under hypoxic conditions. Attempts to identify HIF-1α in equine chondrocytes and human cell lines revealed some interesting data although HIF-1α expression in these cells was not positively confirmed in our studies, probably due to limitations in the available detection reagents. Consistent appearances of protein bands in each culture type may indicate variation of positive HIF-1α protein expressions at least between 2-dimension and 3-dimension systems. However, those proteins may not be the wild type HIF-1α as they were not hypoxia regulated. Apart from HIF-1α, other subunits should be included in the studies of the hypoxia effect.

Chapter VII

GENERAL DISCUSSION

Low oxygen supply causes severe damage to vascularised organs e.g. brain, heart, liver, etc. but this does not occur in non-vascularised tissues including articular cartilage. The avascular nature of this tissue provides a relatively hypoxic and low-nutrient habitat for chondrocytes. However, the level of oxygen that may be defined as 'hypoxia' for highly vascularised tissues may not be able to be applied to articular cartilage. Oxygen tension within cartilage is estimated to be as low as 1% although the exact concentration has never been confirmed. For this reason, in this study 1% oxygen was chosen to represent oxygen tension of cartilage *in vivo* throughout our *in vitro* studies.

In pathologic joints, oxygen levels can fluctuate (Blake et al. 1989) and tend to be lower than in normal joints (Pfander and Gelse 2007). Although oxygen supply may increase following neo-vascularisation within the inflamed joint tissues or because of increasing diffusion through cartilage fissures, there may be a higher metabolic demand of chondrocytes in these conditions. Scar formation after inflammatory processes in synovial membrane may lead to an increased thickness of the overall synovial membrane and an increased distance between vascularised synovial tissue

and articular cartilage, and thus could lead to oxygen insufficiency to chondrocytes and the tissue may be exposed to 'pathologic hypoxia'. Again, the oxygen concentration within pathologic cartilage has never been precisely identified. Due to a lack of information of precise oxygen levels which define physiological and/or pathological hypoxia of chondrocytes, the term 'hypoxia' used to describe our studies is considered equivalent to 1% oxygen (unless stated otherwise).

Maintaining metabolic energy is necessary for every cell type, including chondrocytes in order to survive and function properly. It has been reported that chondrocytes are able to manage cellular energy efficiently even in oxygen scarce environments (Rajpurohit et al. 1996). Our results (**Chapter III**) confirmed their ability to adjust their glucose transportation, which is the first step of glucose utilisation, when they were cultured in low oxygen conditions, by increasing GLUT1 and glucose uptake, possibly to compensate for low ATP production by the glycolytic pathway. Chondrocytes are known to use anaerobic glycolysis as the major energy generating pathway (Lee and Urban 1997); however, it was shown that they switched towards oxidative phosphorylation when they were grown as monolayers (Heywood and Lee 2008). The present glucose uptake assay has a limitation in that it can be applied only to monolayer cultures. Therefore, it is difficult to confirm that our findings reflect the actual metabolic reaction of chondrocytes under hypoxia *in vivo*. An assay which is applicable in 3-D cultures, that allows maintenance of chondrocytic

phenotype to that seen in native cartilage, would be useful in this regard. In addition, to confirm chondrocyte ability to maintain cellular energy under hypoxia, other factors involved in glucose metabolism pathway apart from glucose transportation, eg. glucose utilisation by hexokinase or lactate production, should be included in future studies.

It is known that glucose is not only important for ATP production but also is the substrate for synthesis of UDP-hexoamine and UDP-glucuronic acid (Qu et al. 2007) which are important components of GAGs. If the investigation of glucose transportation using monolayer culture in this study reflected what occurs in native cartilage or 3-D cultures, it is possible that increasing glucose transportation under hypoxic environments may not only be important for energy maintenance but also for matrix synthesis, which is more favourable in chondrocytes in 3-D than in monolayer cultures. However, our findings, in a matrix synthesis study, cannot confirm a greater GAG production in chondrocyte pellet cultures although monolayer culture revealed increased glucose uptake under hypoxia. This could mean that enhanced glucose transportation is probably used mainly for energy maintenance rather than matrix production. This is still yet to be explored in a culture system where phenotype is maintained and which is amenable to the investigation of both glucose utilisation and matrix synthesis.

The pathological conditions of articular cartilage *in vivo* were mimicked by catabolic induction using various human recombinant proinflammatory

cytokines (**Chapter IV**). Here we have, for the first time, investigated the effect of oxygen levels on cytokine-induced cartilage matrix degradation. The results showed that there was no significant alteration of GAG and collagen degradation of cytokine-induced cartilage explants comparing between 20% and 1% O_2. Nonetheless, hypoxia resulted in a trend of reduction of TNF-induced MMP-13 activity. The possibility of catabolic involvement under low oxygen condition by other MMPs, such as MT1-MMP and other collagenases (MMP-1 and MMP-8) was suggested (see **Chapter IV**). However, measuring MMP-13 activity using the available fluorogenic substrate is not entirely specific for MMP-13. MMP-1 and MMP-8 have been shown to cleave this substrate but at lower rates (Knäuper et al. 1996 a). Therefore, it has to be accepted that there might be interference from other MMPs secreted from cartilage explants in this assay. This could have been excluded by using a specific MMP-13 inhibitor (Johnson et al. 2007).

Measurement of TIMPs were also included in the study but only at the mRNA level as an attempt to study TIMP protein and activity by reverse-zymography using MMP-2 and MMP-9 (generated from canine cell lines) was not successful (data not shown). It may be that equine TIMPs are not able to inhibit canine MMPs. For this reason, the balance between MMPs and their inhibitors under hypoxia are not yet fully understood.

Interestingly, from this study (**Chapter IV**), we found that rhTNF-α is the most effective cytokine inducing changes in equine cartilage compared to

the rest of our cytokine panel (IL-1, OSM and combined IL-1 and OSM) because it was able to degrade not only GAG but also collagen in cartilage explants through increased MMP-13 production and activation. This finding is even more interesting when comparing the response of our equine cartilage explants to cartilage explants of other species, i.e. human and bovine, from previous reports (Elliott et al. 2001; Hui et al. 2001; Morgan et al. 2006). In these studies, human and bovine cartilage explants responded well to IL-1 and even better to the IL-1/OSM combination. The explanation for this species variation is still not known. However, it is unlikely that human recombinant IL-1 and OSM were not able to bind to the receptors on equine chondrocyte membrane as it was clearly shown that we could trigger the successful induction of GAG degradation by all human recombinant cytokines including IL-1 and OSM. It may be that equine chondrocytes react differently in cytokine signalling pathways in collagen degradation; this requires further investigation.

TNF-α is known to be able to stimulate intracellular IL-1, which progresses subsequent catabolic pathways (Verbruggen et al. 2007). It is possible that human-recombinant TNF-α triggered the intracellular equine IL-1 and started the cascades of protease activity to degrade collagen. To study this, an IL-1 antagonist would be useful for indicating the role of intracellular IL-1 in conjunction with rhTNF-α. A study in human OA cartilage has demonstrated that blockade of TNF-α and IL-1 using their antagonists could suppress MMP-1, -3 and -13 and therefore reduce type II collagen

degradation and GAG release (Kobayashi et al. 2005). If collagen degradation by rhTNF-α in our cartilage explant model represented an important role for native equine TNF-α catabolic pathways *in vivo*, it would also be interesting to investigate the effect of TNF-α antagonist in clinical equine OA studies.

Adult articular cartilage has a very slow repairing process once it is injured (Eyre et al. 2006). For this reason, regeneration of this tissue is almost impossible. Cartilage tissue engineering is one of the potential approaches to regenerate a homogeneous and functional cartilage matrix. However, over the last decade, there has been an issue whether or not the atmospheric oxygen tension which has been used in chondrocytes cultures *in vitro* is suitable for cartilage neo-construction that is to be clinically applied. The outcome for this debate is still unclear and controversial both from literature surveys and from the results of our studies. In **Chapter V**, we observed the differing anabolic responses of chondrocytes (pellet cultures) from different joints and with differing numbers of passages. Although the actual cause of these confounding findings is not identified, there is one possibility that should not be overlooked. Oxygen gradients may have been generated within the pellets and chondrocytes in the same pellet may be exposed to different oxygen concentrations while the parameters were assessed as an average. Moreover, using gas phase oxygen intervention has a limitation in that it is difficult to confirm the actual oxygen level that the cultures are exposed to after diffusion through

liquid medium (Malda et al. 2004 b). Taken together, the possibility that chondrocytes were exposed to heterogenous oxygen levels may be one of the causes of these inconsistent findings. And it would be interesting to know if the HIF expression pattern in each pellet zone could help to identify the oxygen situation within the chondrocyte pellets or other 3-D constructs.

The recombinant human TGF-β used in our equine cartilage matrix synthesis experiments appeared not to be effective although it has been reported to have chondroinductive effects on human mesenchymal stem cells (Fan et al. 2008) and chondrocytes from other species (Glowacki et al. 2005; Byers et al. 2008). It could be that species variation is an issue, in that recombinant human TGF-β3 may not bind to its receptors on equine chondrocytes or it could be that the concentration of 10 ng/ml TGF-β3 was too high. One study reported that high concentrations (5 and 10 ng/ml) of TGF-β1 provided the best matrix stimulatory effect on a 3-D constructed equine chondrocytes at 7 days of culture period but at 14 days (the same observation point as our study) a lower concentration (1ng/ml) delivered more effective stimulation (Fortier et al. 1997). We do not know whether or not this situation also occurs with TGF-β3 but it will be an interesting issue for the future studies. In addition, recently, a study using bovine chondrocytes on a 3-D construct has reported reproducible and rapid matrix production by using a transient application of TGF-β3 (Byers et al. 2008), which would reduce time and cost in chondrocyte cultures for

matrix synthesis studies compared to continuous TGF-β3 stimulation and may also be applicable to equine chondrocyte cultures.

Hypoxia inducible factor (HIF) is considered a key protein regulating cells under oxygen scarce environments (Wenger 2000). It was included in this study because it could have been a key mediator and explained the data from the studies of glucose transportation and matrix degradation and synthesis under hypoxia. Although a number of studies were able to identify HIF-1α protein expression (Stroka et al. 2001; Lin et al. 2004; Yudoh et al. 2005), we found equine proteins that interacted positively with HIF-1α antibodies but could not confirm its identity in equine chondrocytes (**Chapter VI**). Perhaps chondrocytes express HIF-1α in undetectable levels or there may be a lack of cross reaction between the relevant equine epitope and the antibodies. It is also possible that HIF is rapidly destroyed during the harvesting processes under atmospheric condition. This may be prevented by using a full hypoxic system that allows hypoxic maintenance from harvesting of cartilage throughout the culture experiments or by using HIF stabiliser e.g. dimethyloxalylglycine (DMOG) which can inhibit prolyl-hydroxylases (Irwin et al. 2007).

In retrospect, it would have been valuable to explore the upregulation of HIF-2α and its role in the regulation of responses to hypoxia. Recent studies (Lafont et al. 2007) have shown that the long-term hypoxic

induction of chondrocyte phenotype is driven by sustained upregulation of HIF-2α. This would be very interesting to investigate further in the horse.

Overall, although the key protein regulating cells under hypoxia, HIF, could not be identified, this thesis has demonstrated the ability of chondrocytes to manage energy balance under hypoxic conditions and this is supportive of the hypothesis. However, hypoxia did not result in antagonising matrix catabolism. Indeed it has no significant effect in this regard and hypoxia could not provide a promising positive outcome in matrix production. Based on the findings from this study, there is not enough evidence to confirm that there is a real benefit of low oxygen tension being applied in chondrocyte culture systems.

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LIST OF PUBLICATIONS

1. Peansukmanee S, Vaughan-Thomas A, Carter SD, Clegg PD, Taylor S, Redmond C, Mobasheri A. Effects of hypoxia on glucose transport in primary equine chondrocytes *in vitro* and evidence of reduced GLUT1 gene expression in pathologic cartilage *in vivo*. Journal of Orthopedic Research; In press.

APPENDIX

Effects of Hypoxia on Glucose Transport in Primary Equine Chondrocytes *In Vitro* and Evidence of Reduced GLUT1 Gene Expression in Pathologic Cartilage *In Vivo*

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ABSTRACT: Articular chondrocytes exist in an environment lacking in oxygen and nutrients due to the avascular nature of cartilage. The main source of metabolic energy is glucose, which is taken up by glucose transporters (GLUTs). In diseased joints, oxygen tensions and glucose availability alter as a result of inflammation and changes in vascularisation. Accordingly, in this study we examined the effects of hypoxia and the hypoxia mimetic cobalt chloride ($CoCl_2$) on glucose transport in equine chondrocytes and compared expression of the hypoxia responsive GLUT1 gene in normal and diseased cartilage. Monolayers of equine chondrocytes were exposed to 20% O_2, 1% O_2, $CoCl_2$ (75 μM), or a combination of 1% O_2 and $CoCl_2$. Glucose uptake was measured using 2-deoxy-D-[2,6-^{3}H] glucose. GLUT1 protein and mRNA expression were determined by FACS analysis and qPCR, respectively. GLUT1 mRNA expression in normal and diseased cartilage was analyzed using explants derived from normal, OA, and OCD cartilage. Chondrocytes under hypoxic conditions exhibited a significantly increased glucose uptake as well as upregulated GLUT1 protein expression. GLUT1 mRNA expression significantly increased in combined hypoxia-$CoCl_2$ treatment. Analysis of clinical samples indicated a significant reduction in GLUT1 mRNA in OA samples. In OCD samples GLUT1 expression also decreased but did not reach statistical significance. The increase in glucose uptake and GLUT1 expression under hypoxic conditions confirms that hypoxia alters the metabolic requirements of chondrocytes. The altered GLUT1 mRNA expression in diseased cartilage with significance in OA suggests that reduced GLUT1 may contribute to the failure of OA cartilage repair. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: equine chondrocyte; GLUT1; hypoxia; osteoarthritis; osteochondritis dissecans

Articular cartilage is an avascular tissue. Nutrients and oxygen are delivered by diffusion from blood vessels of the underlying bone and from the synovial fluid.[1] The oxygenation gradients within the tissue have been estimated to be 6–10% at the articular surface to less than 1% O_2 at the deepest layer.[2–7] However, chondrocytes appear to adapt very well to oxygen and nutrient limited environments.[8] Both anabolic activity and cell survival of chondrocytes were found to be maximized when they were cultured in vitro under low oxygen tensions compared to normoxic and anoxic conditions.[9–12]

The main source of metabolic energy in chondrocytes is glucose,[13] which is taken up by a family of substrate specific membrane proteins known as glucose transporters (GLUTs). GLUT isoforms differ in their substrate specificity, tissue distribution, and cellular localization. Articular chondrocytes and intervertebral disc cells express a number of glucose transporter proteins including GLUT1, GLUT3, and GLUT9.[14] GLUT1 and GLUT3 are hypoxia responsive isoforms[15] that are regulated by the transcription factor and oxygen sensor protein, hypoxia-inducible factor 1 (HIF-1).[16,17]

Chondrocytes generate energy mainly by anaerobic glycolysis,[1] which produces 18–19 times less ATP per molecule of glucose than aerobic respiration.[18] Therefore, chondrocytes must be able to manage glucose transport and metabolism effectively to provide sufficient energy within the constraints of what is available in cartilage. Recent studies[19,20] have shown that chondrocytes are capable of maintaining their metabolic balance when deprived of glucose or exposed to the hypoxia mimetic cobalt chloride ($CoCl_2$).

Osteoarthritis (OA) leads to progressive and permanent loss of articular cartilage. Joint injury, the major cause of OA in horses,[21] leads to synovial effusion and increasing vascularization in response to inflammation and healing processes, and this may disturb or alter the oxygen supply within the synovial environment.[22] Osteochondritis dissecans (OCD) is categorized as a developmental orthopedic disease, involving a failure of endochondral ossification, which is associated with a failure of vascular invasion from the subchondral bone and leads to development of cartilage lesions that can become detached at a later date.[21] The ischemia–reperfusion from the neovascularization process in traumatic joints before OA development[22] and the ischemic necrosis of the subchondral bone during OCD formation may cause fluctuations in oxygen levels and result in metabolic imbalance by disturbing glucose transport.

The aim of this study was to test the hypothesis that chondrocytes are able to adapt their energy metabolism under hypoxia, using low oxygen (1% O_2) or exposure to a hypoxia mimetic (cobalt chloride). We also investigated whether expression of a key hypoxia responsive glucose transporter (GLUT1) varied in normal cartilage comparing to degenerative cartilage, such as found in osteoarthritis (OA) and osteochondritis dissecans (OCD).

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MATERIALS AND METHODS

Reagents

All tissue culture reagents were from Invitrogen (Carlsbad, CA). Polyclonal antibodies against GLUT1 were donated by Dr. S.A. Baldwin (University of Leeds, UK). The GLUT1 antiserum was developed in rabbits against the C-terminus of rat-GLUT1 (residues 477–492). We have previously confirmed that this GLUT1 antibody recognizes GLUT1 across a diverse number of mammalian species including rats, humans, dogs, sheep, and horses.[23,24] In this study we used this GLUT1 antibody in Western blots of equine chondrocyte lysates to confirm positive immunoreactivity with a band migrating at 45–50 kDa (the expected molecular weight for GLUT1[25]) (data not shown).

Cartilage Source

Samples were collected in accordance with institutional guidelines with ethical review consent and written informed consent from the animal owners. Normal equine articular cartilage was obtained from the trochlear ridges and medial femoral condyles of the femur of horses aged between 2–12 years old, euthanased for unrelated clinical reasons at the Philip Leverhulme Large Animal Hospital, University of Liverpool. OCD cartilage biopsies were harvested from the lateral trochlear ridge of the femur (LTRF) from five OCD horses (aged 4–7 years) during therapeutic arthroscopic debridement of the lesions. OA cartilage biopsies were harvested immediately postmortem from the medial femoral condyle of eight horses that were euthanased as a consequence of lameness due to severe osteoarthritis of the medial femorotibial joint with confirmation of OA by gross postmortem and histological analysis in all cases. Where tissue was harvested for mRNA isolation, the cartilage was placed in RNAlater™ and then transferred on ice to the laboratory for further processing.

Chondrocyte Isolation and Culture

Cartilage was rinsed with Dulbecco's modified Eagles medium (DMEM) with 300 units/mL penicillin G sodium, 300 μg/mL streptomycin, and 7.5 μg/mL amphotericin B, cut into small pieces, and digested overnight with 0.1% type I collagenase (EC 232.582.9 from *Clostridium histolyticum*) at 37°C. Isolated cells were washed and cultured as a monolayer in DMEM with 1 g/L glucose, L-Glutamine, and pyruvate, supplemented with 10% fetal calf serum, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. All cell culture experiments were performed on first passage chondrocytes (the first passage after the expansion phase in monolayer; cells were seeded at 5000 cells/cm^2) and used in experiments when they were approximately 80% confluent (estimated by light microscopy).

2-Deoxy-D-[2,6-^{3}H] Glucose (2-DOG) Uptake

Net glucose transport was determined by measuring the uptake of nonmetabolizable 2-deoxy-D-[2,6-^{3}H] glucose (Amersham Biosciences, Piscataway, NJ) using the protocol previously published.[24,26] Monolayer equine chondrocytes were preconditioned in 20% or 1% O_2 with or without 75 μM $CoCl_2$ for 48 h. The glucose uptake assay was then performed using 0.5 μCi/mL 2-DOG in modified DMEM lacking glucose, pyruvate, and serum at 37°C for 2 h with a presence or absence of the glucose transport inhibitor, cytochalasin B (20 μM). Nearly 100% chondrocyte viability was confirmed in a separate study. Scintillation counts in each sample were normalized to protein content using the Lowry protein assay.[27]

Fluorescent Activated Cell Sorting (FACS) Analysis of GLUT1 Expression

Immunostaining and FACS analysis were carried out essentially as described previously.[14] Nearly confluent first passage monolayer equine chondrocytes were maintained under 20% O_2 or 1% O_2 for 48 h. Trypsinized cells were washed and fixed with 3.7% paraformaldehyde for 10 min. Cells were permeabilized with 0.05%Triton X-100 in PBS for 10 min at 4°C and incubated with anti-GLUT-1 polyclonal antibody, which has previously been shown to crossreact with equine chondrocytes by Western blot,[24] at the dilution of 1:600 in PBS, followed by goat antirabbit IgG conjugated with FITC (Sigma, St. Louis, MO) at 1:40 in PBS, each for 30 min at 37°C in light protective containers. Immunolabeled chondrocytes were washed and resuspended in PBS before being analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) by gating 10,000 cells. The detectors were set as follows: FCS (threshold): 200; FSC: E-01; SSC: 319; and FLI: 408, and the parameters were: FSC-H: 5.0; SSC-H: 1.2; and FL-1 H: log. The data were analyzed by WinMDI 2.8 (free downloadable software; http://facs.scripps.edu/software.html) to obtain (1) number of cells expressing fluorescence and (2) mean fluorescent intensity (MFI), which defines an average GLUT1 expression in each sample.

RNA and cDNA Preparation

Total RNA was extracted from chondrocyte monolayers using TRIzol® Reagent (Invitrogen) and purified through RNeasy® columns with DNaseI (Qiagen, Chatsworth, CA), according to the manufacturer's instructions, to remove genomic DNA. cDNA copies were made from 1.5 μg RNA using oligo (dT) primer (Promega, Madison, WI) and the reverse transcriptase from Invitrogen or Promega. The synthesized cDNA were kept at −20°C until use. The same protocol was applied for cartilage explant RNA and cDNA preparation from normal, OA, and OCD cartilage biopsies but the explants were snap-frozen and pulverised in liquid nitrogen cooled steel chambers (Braun Biotech Mikrodismembrator, Allentown, PA) prior to the RNA extraction.

Reference Gene Selection and Quantitative Polymerase Chain Reaction (qPCR)

The candidate reference genes and their primers are shown in Table 1. They were selected on the basis that they belong to different functional classes.[28] Equine RNA and DNA sequences were sourced from the NCBI database (http://www.ncbi.nlm.nih.gov/). If the equine sequence was unavailable then a multiple species alignment was carried out (www.ebi.ac.uk/Tools/clustalw/) and primers designed where there was sequence homology. Human exon boundaries were identified using the Ensembl Genome Browser (http://www.ensembl.org/index.html), and where possible, primers were designed to cross predicted exon boundaries. The primers were designed based on the above sequences using Primer Express (Applied Biosystems, Bedford, MA) software or Roche (Indianapolis, IN) primer design (https://www.roche-applied-science.com/). Primer efficiencies were validated using a standard curve derived from equine tendon cDNA (a 10-fold dilution series with five measuring points).

Expression of all seven candidate reference genes were analyzed across the chondrocyte monolayer cultures ($n = 5$) exposed to 20% O_2 or 1% O_2 with and without 75 μM $CoCl_2$ or 100 ng/mL IL-1β for 24 h. Another analysis was done across normal ($n = 6$), OA ($n = 8$), and OCD ($n = 5$) cartilage to investigate the expression of five candidate genes (HPRT-1, TBP, GAPDH1, GNB2L1, and 18S). The most stable pair of

Table 1. Candidate Reference Gene List

Symbol	Gene Name	Accession Code	Primers 5′-3′	Efficiency on Equine Tendon	References
GAPDH (1)	Glyceraldehyde-3-phosphate dehydrogenase	AF157626	Fw: GCATCGTGGAGGGACTCA Rv: GCCACATCTTCCCAGAGG	−3.317	Roche
GAPDH(2)	Glyceraldehyde-3-phosphate dehydrogenase	AF157626	Fw: TGACCCCCTAACATATTGAGAGTCT Rv: GCCCCTCCCCTTCTTCTG	−3.139	Primer Express
ACTB	Actin, beta	AF035774	Fw: CCAGCACGATGAAGATCAAG Rv: GTGGACAATGAGGCCAGAAT	−3.333	Bogaert et al, 2006[5]
TBP	TATA box binding protein	NM_001075742	Fw: TGCTGCTGTAATCATGAGGGTAA Rv: TCCCGTGCACACCATTTTC	−3.54	Primer Express
HPRT-1	Hypoxanthine phosphoribosyltransferase I	AY372182	Fw: GGCAAAACAATGCAAACCTT Rv: CAAGGGCATATCCTACGACAA	−3.35	Bogaert et al, 2006[5]
18S	Ribosomal protein, 18S	AJ311673	Fw: GGCGTCCCCCAACTTCTTA Rv: GGGCATCACAGACCTGTTATTG	−3.241	Primer Express
GNB2L1	Guanine nucleotide binding protein	NM_006098	Fw: CCTTGTGCTTCAGTCCCAAT Rv: CAATGATCTTGCCCTTCAAGT	-3.241	Roche

genes was identified using two downloadable software programs; GeNorm (http://medgen.ugent.be/~jvdesomp/genorm/) and Normfinder (http://www.mdl.dk/publicationsnormfinder.htm), according to the software instructions. PCR reactions were performed in a 10 μL volume using SYBR® Green PCR master mix (Applied Biosystems) and 300 nM primer concentration and processed by 7900HT Fast Real time PCR system (Applied Biosystems) using standard amplification conditions. The data were analyzed by SDS software (v 2.2.1; Applied Biosystems). The pair of reference genes that had the most consistent expression, represented by the lowest stability value, was selected. The mRNA expression of GLUT-1 was assessed using monolayer cultures ($n = 3$) of equine chondrocytes exposed to 20% O_2 or 1% O_2 with and without 75 μM $CoCl_2$ for 24 h and the cartilage explants from normal ($n = 6$), OA ($n = 8$), and OCD ($n = 5$) horses. The qPCR was performed with the same protocol described above using the following primers: forward primer; 5′-AGCAGCCTGTGTACGCCAC-3′ and reverse primer; 5′-CTCGTTCCACCACAAACAGC-3′. These primers were designed by the Primer3 software (http://primer3.sourceforge.net/) using publicly available sequences from the NCBI Database. PCR products were measured and normalized against the selected reference genes.

Statistical Analysis

One-way analysis of variance (ANOVA) with Dunnett's multiple comparison test was performed by GraphPad Prism software (v.4.01) to compare treatment or diseased groups with the control or normal groups, respectively.

RESULTS

2-Deoxy-D-[2,6-^{3}H] Glucose Uptake

Figure 1 shows the alteration of 2-DOG transport rates. The data are represented as percentage of the control (100% ± 0.123 pmol/min/mg protein). The uptake was significantly increased ($p < 0.05$) both in hypoxia (1% O_2) and $CoCl_2$. An additive effect of the combined 1% O_2 and $CoCl_2$ was observed ($p < 0.01$). Cytochalasin B effectively inhibited 2-DOG uptake ($p < 0.01$).

Fluorescent Activated Cell Sorting (FACS) Analysis

FACS analysis indicated that approximately 90% of all groups of chondrocytes expressed GLUT1 protein (Table 2) with a confirmation by statistical analysis that there was no significant difference between normoxia and hypoxia treatments. However, the intensity of GLUT1 protein expression, which was represented as mean fluorescent intensity (MFI), in chondrocytes exposed to 1% O_2 or $CoCl_2$ was significantly increased (Fig. 2) ($p < 0.05$). The combination of those two treatments had a tendency of increasing

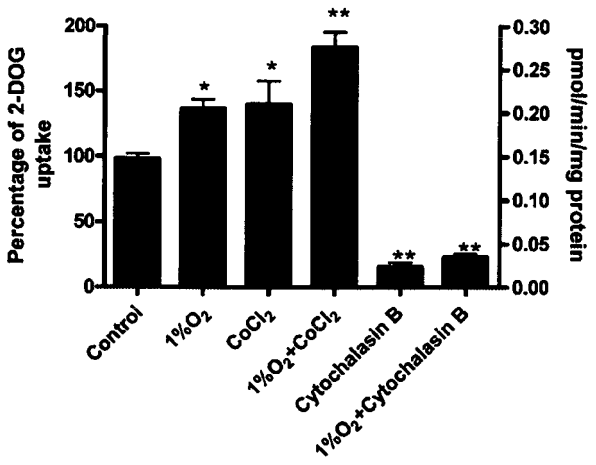

Figure 1. Effect of hypoxia (1% O_2), cobalt chloride (75 μM), and the combined treatment on 2-deoxy-*D*-[2,6-^{3}H] glucose uptake by first passage equine articular chondrocyte monolayers ($n = 3$). The cultures were preconditioned for 48 h in such treatments prior to exposure to 0.5 μCi/mL in glucose and pyruvate free DMEM at 37°C for 2 h in the presence and absence of 20 μM cytochalasin B. Results represent percentage glucose uptake to the control group (20% O_2) (average uptake in the control group was taken as 100%, which is approximately 0.123 pmol/min/mg protein). The error bars represent SEM.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 2. Number of Chondrocytes (Mean, Standard Deviation, and Standard Error) That Expressed GLUT1 (from 10,000 Gated Cells)

Treatment	Mean	Standard Deviation	Standard Error
Control	8997.33	± 1246.55	± 415.52
Hypoxia (1% O_2)	9300.78	± 1335.25	± 445.08
75 μM $CoCl_2$	9427.89	± 740.45	± 246.82
Hypoxia + $CoCl_2$	9419.22	± 856.50	± 285.50

GLUT1 expression compared to the control but this was not significant.

Reference Gene Selection for qPCR Analysis

The real-time PCR assays were designed to detect and quantify expression of commonly used reference genes (Table 1). The stability of gene expression across different samples was analyzed using GeNorm and Normfinder software. The stability values and the ranking of the candidate genes from both forms of analysis in the study with chondrocyte cultures and a study of normal versus diseased cartilage study are shown in Table 3. Although the ranking of the candidate genes was somewhat different between the two programs, HPRT-1 and TBP were identified, in both studies and both analyses, to be the most stably expressed pair of genes and were selected to use as the reference genes for the rest of this study. Normfinder identified GAPDH(1) as the best single reference gene in the normal-disease cartilage tissue study.

GLUT1 mRNA Expression

The relative expression of GLUT1 mRNA was quantified by real-time PCR assay. The Ct values of all samples were normalised with the mean Ct values of the selected reference genes, which were HPRT-1 and TBP. There was an upregulation of the GLUT1 mRNA expression in samples exposed to 1% O_2 and 75 μM $CoCl_2$; however, only a combination of both treatments showed a statistically significant increase ($p < 0.05$) (Fig. 3a). In contrast, examination of GLUT1 mRNA expression in cartilage tissue, as shown in Figure 3b, identified that in OA cartilage its abundance was significantly decreased ($p < 0.01$) compared to normal cartilage. In OCD samples, expression of GLUT1 was also decreased but this reduction did not reach statistical significance.

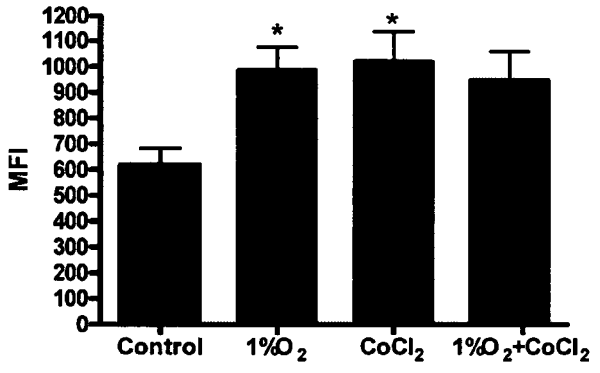

Figure 2. Changes in equine chondrocyte GLUT1 protein expression assessed by FACS analysis using first passage equine chondrocytes in monolayer culture after exposure to hypoxia (1% O_2), Cobalt Chloride (75 μM) and the combined treatment for 48 h. The results the represented as mean fluorescent intensity (MFI). The experiment was done with three different cartilage donors. The error bars represent SEM.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

DISCUSSION

In this study we observed enhanced glucose uptake by equine chondrocytes in a low oxygen environment and a highly significant increase in glucose transport in hypoxia combined with hypoxia mimetic, suggesting an increased metabolic demand of the chondrocytes in response to hypoxia. This is supportive of previous studies in equine chondrocytes using $CoCl_2$.[19] The inhibition of glucose uptake by cytochalasin B indicates that the glucose uptake is mediated by sodium-independent glucose transporters.[29] Glucose transporter 1 (GLUT1) is known to be the most abundant GLUT expressed by most cell types, including articular chondrocytes and intervertebral disc cells.[17,30] GLUT1 is generally considered to function as a "housekeeping" glucose transporter and in this role, GLUT1 maintains basal glucose uptake for metabolic reactions.[13] GLUT1 is also reported to be a "hypoxia-responsive" glucose transporter in various tissues,[20,31,32] especially in cancer cells that collectively exhibit the Warburg effect (reviewed in ref. 33). This observation was confirmed by our investigation of chondrocyte GLUT1 protein expression. We found that although most of the chondrocytes examined expressed some GLUT1 protein, the physical (1% O_2) and chemical ($CoCl_2$) hypoxia signals significantly increased the amount of GLUT1 that is expressed in each chondrocyte.

However, the glucose uptake results were not fully concordant with the GLUT1 protein expression data, especially in chondrocytes exposed to a combination of hypoxia and $CoCl_2$. Although glucose uptake showed an additive effect of the combined hypoxic treatment, GLUT1 protein expression was more or less at the same level as individual 1% O_2 and $CoCl_2$ treatments. One plausible explanation for this observation is an increase in the translocation of preexisting GLUT1 protein from subcellular compartment to the cell membrane[20] in an extremely hypoxic environment. It is also possible that other glucose transporters, such as GLUT3 and GLUT9, which have recently been shown to be expressed in chondrocytes,[14,34] are involved in this process. GLUT3 is known to be a high-affinity glucose transporter,[13] and could have a role in regulating glucose transport under such stimuli where chondrocytes have a very high metabolic demand to maintain their matrix synthesis function. Furthermore, GLUT3 has been found to be upregulated in a number of hypoxic tumours along with GLUT1 (reviewed in ref. 33). We could not identify

Table 3. Stability Value and Ranking of Candidate Reference Gene Expression

Experiment	Gene	Normfinder Stability Value	Rank	Gene	GeNorm Stability Value	Rank
Equine chondrocyte cultures	HPRT-1[a]	0.195	1	HPRT-1	0.244047225	1
	TBPα	0.204	2	TBP	0.244047225	2
	GNB2L1	0.244	3	GAPDH(1)	0.54765329	3
	18S	0.283	4	GAPDH(2)	0.560551071	4
	GAPDH (1)	0.336	5	GNB2L1	0.602052909	5
	GAPDH (2)	0.378	6	18S	0.650220526	6
	ACTB	0.451	7	ACTB	0.712836738	7
Normal and diseased cartilage explants	GAPDH (1)	0.048	1	HPRT-1	0.643906	1
	HPRT-1[a]	0.055	2	TBP	0.643906	2
	TBP[a]	0.058	3	GAPDH(1)	0.728417	3
	GNB2L1	0.117	4	GNB2L1	0.890601	4
	18S	0.141	5	18S	1.248013	5

[a]The best combination of two genes by Normfinder.

equine GLUT3 and GLUT9 gene sequences, and the prediction of primer sequences from other species was not successful. Therefore, GLUT3 and GLUT 9 qPCR could not be performed in this study. Another alternative to explain this phenomenon is perhaps changes in hexokinase activities as there has been evidence of alterations in hexokinase expression and activities by hypoxia.[35,36]

We successfully investigated the expression of the GLUT1 gene in response to low oxygen tension and the presence of cobalt chloride, using quantitative, real-time PCR. Although GLUT1 protein expression was significantly higher in hypoxic conditions, mRNA expression, while elevated, did not reach statistical significance. The smaller response in GLUT1 mRNA expression to hypoxia or $CoCl_2$ stimulation suggests that increasing the expression of GLUT1 gene may not be the main mechanism used by chondrocytes to regulate glucose metabolism under a low oxygen environment; other mechanisms may be involved including increased GLUT1 mRNA stability and posttranslational regulatory mechanisms (i.e., recruitment and activation of preexisting GLUT1 transporters). Zhang and colleagues[20] have reported that the acute response to hypoxia is mainly mediated by enhanced function of the existing glucose transporters, especially GLUT1, while the regulation at transcriptional level of GLUT1 is found to increase in chronic hypoxia. However, our data did show that the combination of hypoxia and $CoCl_2$ treatment caused a reproducible and significant increase in GLUT1 mRNA levels, indicating that the transcription of GLUT1 can be significantly increased acutely, dependent on the relevant stimuli.

The reference gene selection was addressed in our studies as it is reported that hypoxia is able to alter the

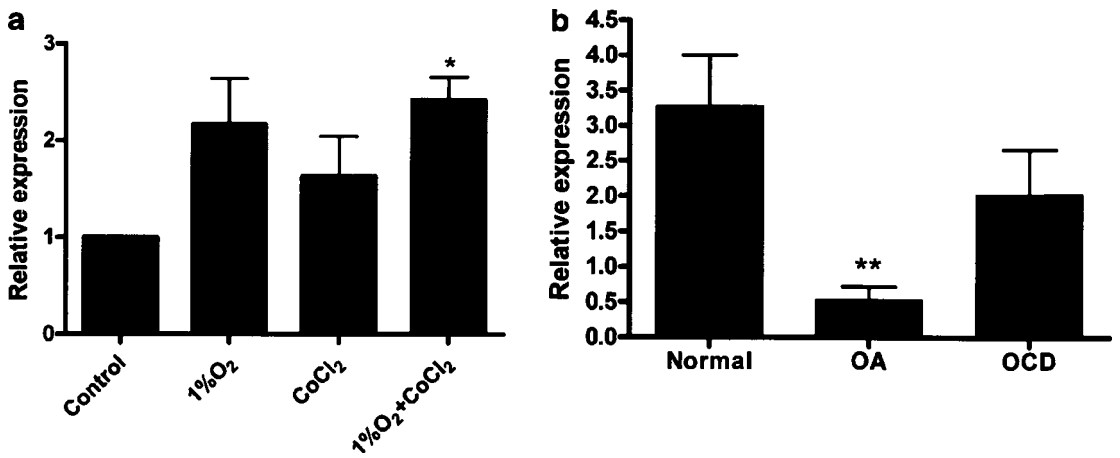

Figure 3. GLUT1 mRNA expression (a) study in first passage equine chondrocyte monolayers after exposure to normoxia (20% O_2), hypoxia (1% O_2), and/or 75 μM cobalt chloride for 24 h using cartilage from five different donors, and (b) study in normal ($n = 6$), osteoarthritis (OA) ($n = 8$), and osteochondritis dissecans (OCD) ($n = 5$) cartilage explants. The analysis was done in triplicate. The error bars represent SEM.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[37] which has been commonly used as a reference gene in many chondrocyte quantitative PCR analyses. A reference gene selection study was therefore performed. Two candidate reference genes were selected for normalization of GLUT1 mRNA expression in each sample. These were HPRT-1 and TBP, and are likely to be the most useful reference genes for all future studies of mRNA experiment by chondrocytes. However, it should be noted that GAPDH, a commonly used reference gene, was shown to be the single most stably expressed gene in our panel of assessed genes for mRNA quantification of *in vivo* acquired cartilage. This is perhaps not surprising given the central role that GAPDH plays in glucose utilization and cellular metabolism.

In the second part of our study, we focused on the expression of GLUT1 mRNA in equine joint diseases. We compared GLUT1 expression in normal cartilage and cartilage derived from joints with either OA or OCD. Interestingly, our findings indicated decreased GLUT1 gene expression in OA and OCD specimens, with a greater reduction and statistical significance in the OA samples, suggesting that glucose transport is affected in diseased joints, especially in OA chondrocytes. The observed reduction of GLUT1 transcription may be a consequence of fluctuations in oxygenation and nutrient supply caused by systemic effects or disturbances in the vascular supply during pathological processes.[22] However, HIF-1, the key protein potentially linking oxygen tension and GLUT1 expression in equine chondrocytes, has not yet been investigated in this species. There is evidence of increased expression of HIF-1α and GLUT1 protein in human OA cartilage, possibly in order to compensate for the increase in ATP demand.[38] In contrast, we observed downregulation of equine GLUT1 mRNA in equine OA cartilage, suggesting failure to maintain energy supply in equine degenerative cartilage.

In summary, hypoxia and the osteoarthritic process have significant effects on glucose transport and GLUT1 expression in chondrocytes. Hypoxia increases the net rate of glucose uptake and the regulation of GLUT1 expression while OA and OCD lead to a reduction in transcription of the GLUT1 gene. The altered GLUT1 mRNA expression in diseased cartilage suggests that there may be a failure in the maintenance of adequate levels of metabolic and structural glucose in OA cartilage. This may contribute to and exacerbate the poor cartilage repair that is characteristic of degenerate cartilage tissue in OA.

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