Oxygen and pH-sensitivity of articular chondrocytes

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By

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Abstract

Articular chondrocytes reside in a unique and challenging avascular environment in cartilage. During joint disease, O_2 and pH levels are further reduced, and may be involved in chondrocyte dysfunction and cartilage matrix breakdown. How fluctuations in these environmental parameters affect factors such as redox balance and mitochondrial function in articular chondrocytes is largely unknown but may provide a link between the extracellular environment to cell signalling pathways that may alter cellular and hence cartilage integrity. Additionally, ROS-regulatory compounds may offer scope for therapeutic intervention in joint disease.

In this project, the effects of different O_2 levels (<1%, 2%, 5% or 21% O_2), pH (pH 7.2 or pH 6.2) and exposure to the pro-inflammatory cytokine, interleukin-1β (10ng/ml) on cell viability and glycosaminoglycan (GAG) release, redox balance (ROS production, GSH:GSSG ratio, NO release, SOD expression) and mitochondrial function (mitochondrial membrane potential ($\Delta\Psi m$)) in articular chondrocytes cultured in 3-D alginate beads were analysed. The ability of the ROS regulatory compounds N-acetylcysteine (NAC) (2mM) and resveratrol (10μM) to alter these variables was also assessed.

This study demonstrates the distinct and interactive effect of O_2 tension and pH to influence articular chondrocyte redox balance and mitochondrial function. Cellular ROS levels, $\Delta\Psi m$ and SOD expression all displayed O_2 sensitivity, being reduced by hypoxia/anoxia. Articular chondrocytes also displayed pH sensitivity with acidosis modulating ROS levels, $\Delta\Psi m$ and GSH content. Combined conditions (hypoxia/anoxia and acidosis) accentuated responses and further compromised chondrocyte integrity (survival, GAG release, phenotype expression). Treatment with NAC and resveratrol altered these variables through distinct mechanisms and conferred protection against O_2 and acid-mediated oxidative stress. Both NAC and resveratrol abolished anoxic-induced inhibition of SOD levels. NAC significantly abrogated anoxic and acidosis-induced GSH inhibition, whereas resveratrol modulated ROS levels and restored O_2 and pH mediated mitochondrial depolarisation.

This thesis details the powerful effect of the cellular environment to modulate chondrocyte function. Manipulation of this environment or targeting related molecular pathways may hold promise for alleviation of disease-associated oxidative stress and are discussed.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

γ-GCS	Gamma-glutamyl cysteine ligase
ΔΨm	Mitochondrial membrane potential
μM	Micromolar
6-CF	6-carboxyfluorescein
6-CFDA	6-carboxyfluorescein diacetate
ADP	Adenosine di phosphate
AM	Acetoxymethyl
ANOVA	Analysis of variance
AOX	Antioxidant
ATP	Adenosine tri phosphate
ATP_i	Intracellular ATP
BCA	Bicinchoninic acid
BCECF-AM	2’, 7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaCl	Calcium chloride
CAT	Catalase
COL2A1	Collagen type II
COMP	Cartilage oligomeric matrix protein
COX-2	Prostaglandin-endoperoxide synthase 2
CVD	Cardiovascular disease
DCF	2’ 7 ′ -dichlorofluorescein
DCFH	2 ‘ 7 ‘ -dichlorodifluorescein
DCFH-DA	Dichlorofluorescin diacetate
ddH_2O	Double distilled water
DMEM	Dulbecco’s Modified Eagles Medium
DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EGTA	Ethylene glycol tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ETC	Electron transport chain
EQHAC	Equine hyaline articular chondrocyte
GAG	Glycosaminoglycan
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Reduced glutathione
GSSG	Oxidised glutathione

H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3O^+	Hydronium ion
HAC	Hyaline articular chondrocyte
HBSS	Hanks buffered salt solution
HCL	Hydrogen chloride
HCO_3^-	Bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HOAC	Osteoarthritic hyaline articular chondrocyte
IL-1-β	Interleukin 1 beta
IMM	Inner mitochondrial membrane
K^+	Potassium
KCl	Potassium chloride
KEAP1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
$MGSO_4{}_6H_2O$	Hexahydrite
mM	Millimolar
mOsm	Milliosmole
MMP	Matrix metalloprotease
MOPS	3-(N-morpholino) propanesulfonic acid
N_2	Nitrite
N_3	Nitrate
Na^+	Sodium
Na^+/k^+ (NHE)	Sodium/hydrogen exchanger
NAC	N-acetyl-cysteine
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NRF-2	Nuclear factor (erythroid derived 2) like-2
nM	Nanomolar
NO	Nitric oxide
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
OA	Osteoarthritis
PBS	Phosphate buffered saline
PGAM5	Phosphoglycerate mutase family member 5
PGE2	Prostaglandin E2
pH_i	Intracellular pH
pH_o	Extracellular pH

PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
RA	Rheumatoid arthritis
RCF	Relative centrifugal force
RES	Resveratrol
RLU	Relative light unit
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS-PAGE	Sodium Dodecyl sulphate-polyacrilamide gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
WR	Working reagent

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UTT

Chapter 1

Introduction

1.1. Articular cartilage structure and function

Articular cartilage consists of an aneural and avascular matrix that forms a smooth, thin layer of highly specialised tissue which covers the surface of diathrodial joints (Poole, 1997). As a result, waste products and nutrients must diffuse throughout cartilage to contribute to cellular processes (Gibson et al, 2008). Chondrocytes are the only cell type that reside within articular cartilage and are crucially responsible for the synthesis and preservation of the extracellular matrix (ECM) in health and disease (Lafont, 2010). Articular cartilage has the critical function of distributing dynamic and static biomechanical load, permitting frictionless motion and maintaining shearing forces in the joint. The complex structure and composition of articular cartilage allows this tissue to fulfill these unique properties. Along with high water content, mature articular cartilage matrix can be divided into two fundamental components: the fibrillar matrix and the extrafibrillar matrix.

The fibrillar matrix component (~50-60% of dry weight) predominantly consists of type II collagen fibres (~95% total tissue collagen) along with type XI (~3%) and type IX (~1%) collagen, forming a dense, crosslinked, heteropolymeric collagen template responsible for cartilage shape, form and counteracting periodic expansion from the hydrophilic aggrecan molecules. Crucially, this provides cartilage with tensile strength (Aigner & Mckenna, 2002; Martell-Pelletier et al, 2008). All collagens are comprised of regions which contain 3 polypeptide chains (glycine and proline), stabilised by hydroxyproline and hydrogen bonds that decorate the length of the molecule. Each polypeptide chain is wound into a triple helix structure which gives the matrix its well documented tensile properties (Fox et al, 2009). Collagen type II molecules are joined together by crosslinks from one collagen telopeptide to the helical structure of another, forming collagen fibrils, which are resistant to proteolytic degradation. Each fibril may contain hundreds of collagen molecules across its diameter. Mature collagen undergoes little turnover over a lifespan under normal conditions, with calculations placing the half-life of mature collagen at 117 years (Martell-Pelletier et al, 2008; Verzijl et al, 2000). They are however cleaved by collagenases such as metalloproteases and

gelatinases as seen in joint diseases such as osteoarthritis (OA), but this is a slow process due to the complexity of crosslinks and number of collagen molecules per fibril (Martell-Pelletier et al, 2008).

The extrafibrillar matrix is comprised mainly of proteoglycans (~35% of dry weight), predominantly the highly sulphated and viscoelastic aggrecan, but also other smaller proteoglycans (biglycan, decorin, fibromodulin) and non-collagenous proteins (fibronectin, cartilage oligomeric matrix protein (COMP) (Aigner & Mckenna, 2002). Aggrecan consists of glycosaminoglycan (GAG) side chains covalently attached to a core protein. Aggrecan associates with hyaluronic acid, being attached by the link protein to form large proteoglycan aggregates (~100 aggrecan molecules in length) that exist within zones of the collagen network (Fox et al, 2009; Martell-Pelletier et al, 2008) (Figure **1.1**). Proteoglycan aggregate structures are less stable than the collagen fibrillar network (half-life of ~1.5 years in normal mature cartilage (Verbruggen et al, 1990)) and hyaluronic acid, aggrecan and link protein integrity are regularly turned over during an individual's lifespan, being strongly regulated by catabolic enzymes such as aggrecanases and matrix metalloproteases (Martell-Pelletier et al, 2008).

The GAG chains of aggrecan are largely made up of keratin and chondroitin sulphate chains which contain thousands of negatively charged molecules attached to the protein core (Dudhia, 2005). This high charge attracts water and hydrates the proteoglycan aggregate (via the Donnan equilibrium), and is thus osmotically active. Swelling of proteoglycan aggregates due to the polyanionic attraction of water is increased during compressive force and is resisted by the inflexible collagen fibrils which creates pressure and allows the cartilage to deform reversibly under load (Dudhia, 2005). If the swelling pressure exceeds the osmotic pressure, water is forced out of the ECM, lubricating the surface of the joint, a key property of articular cartilage (Dijkgraaf et al, 1995; Dudhia, 2005). This system is reliant on a high GAG content within the ECM. Reductions in aggrecan or GAG, such that are seen in ageing cartilage, ultimately lead to cartilage tissue degeneration due to changes in biomechanical load which increases protease secretion and disordered cellular responses (Martell-Pelletier et al, 2008). As

aggrecan has unique hydration properties, it also plays a major role in nutrient transport. Movement of water during joint loading essentially stirs the fluid around the ECM supplying nutrients throughout the tissue (Martell-Pelletier et al, 2008; Zhou et al, 2004). Together, the cartilage ECM is considered to behave as a 'string and balloon' model whereby the hydrated proteoglycans are restricted by the dense collagen network (Kaab et al, 1998).

The fibrillar and extrafibrillar components of cartilage are ordered into four regions, from the cartilage surface to the subchondral bone: the superficial zone, the transitional zone, the deep zone and the calcified cartilage zone. Each zone displays different physical and structural characteristics allowing for optimal function. Between the first three zones, there are no specific boundaries by which to distinguish zones apart. Chondrocytes, however, exert heterogeneity and have altered functions within zones which are dependent on their position and location within the cartilage matrix (Martell-Pelletier et al, 2008) (Figure **1.2**). As is the nature of the articulating joint, cartilage experiences intermittent weight loading, and thus, changes in the physicochemical environment within the tissue are commonplace. Fluctuating osmolarity, hypoxia and reduced pH create an environment unsuitable for most cells. For the chondrocyte however, such environments are a requisite for cellular homeostasis and maintenance of matrix synthesis (Gibson et al, 2008). Immobilisation for instance, and therefore modifications to normal loading patterns leads to changes in ECM metabolism, typified by irreversible proteoglycan loss (Jortikka et al, 1997).

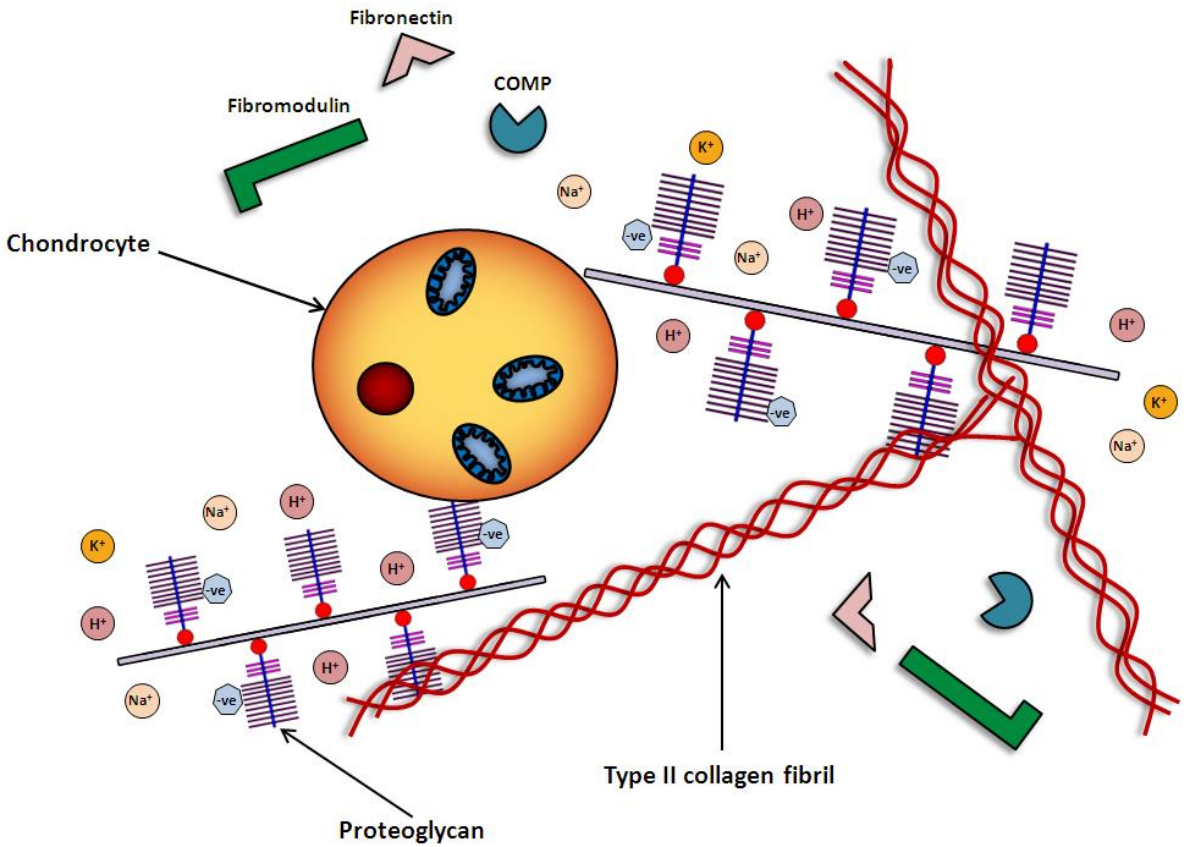


Figure 1.1: Interactions of ECM proteins that comprise the articular cartilage. Collagenous proteins (collagen type II), non-collagenous proteins (e.g. fibronectin, COMP), proteoglycans and small leucine rich proteoglycans (e.g. fibromodulin) create the ECM of hyaline articular cartilage. Negatively charged proteoglycans and rigid type II collagen fibres provide the structural properties associated with the articulating forces of the joint.

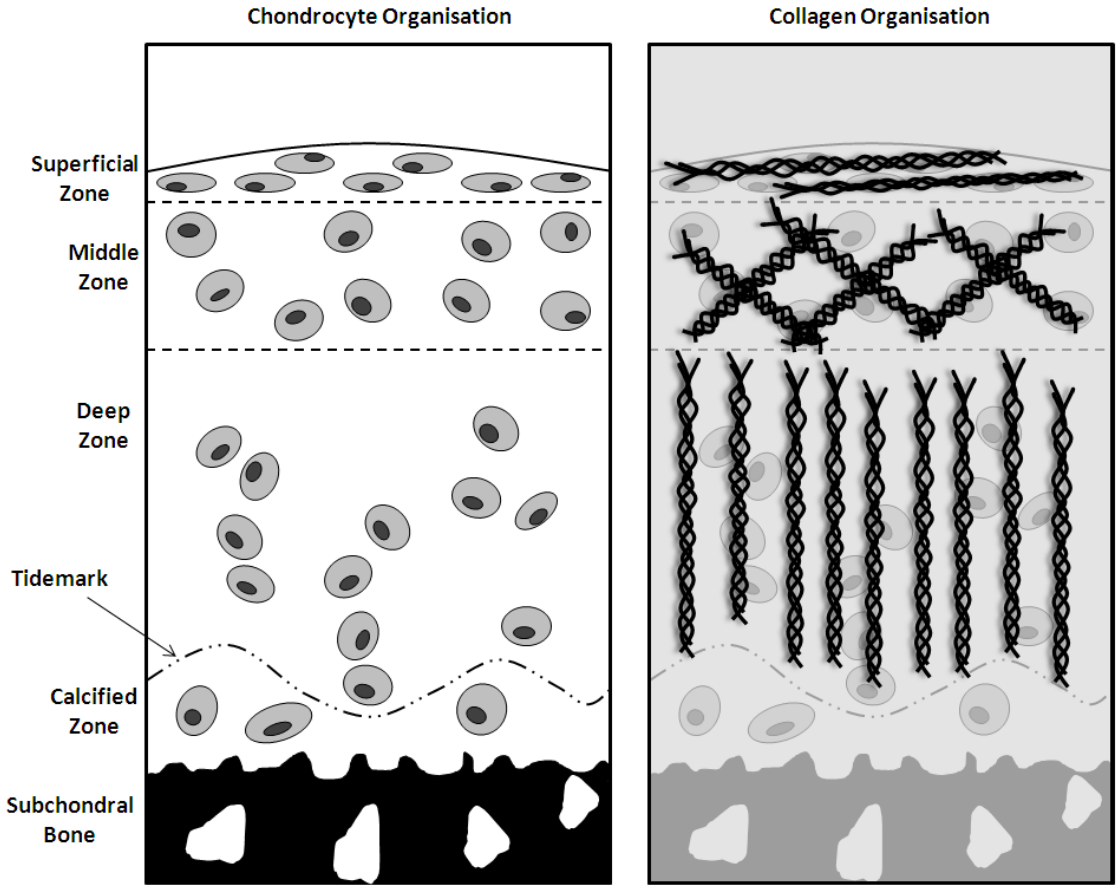


Figure 1.2: Chondrocyte and collagen organization and distribution within different zones of articular cartilage. The superficial zone lies in contact with the synovial fluid and confers tensile properties associated with articulating joint forces. This zone contains few proteoglycans, a thin layer of dense collagen fibrils and flat, elongated chondrocytes orientated parallel to the cartilage surface. The transitional (middle) zone contains fewer chondrocytes which are larger and spherical with a random distribution. Aggrecan is abundant and with thick decussating collagen fibrils, forms layers of tissue that correspond to up to 60% of overall cartilage height. In the deep zone aggrecan content is high (providing resistance to compressive force) and chondrocytes are spherical, arranged in columns and low in density. Collagen fibrils are radially distributed and are structurally largest in this zone. Calcified cartilage anchors the cartilage to the bone via collagen fibrils that penetrate from the deep zone to the calcified cartilage. Chondrocytes are hypertrophic and are physically separated from the deep region by the 'tidemark' (Martell-Pelletier et al, 2008; Poole, 1997).

1.2. The articular chondrocyte and the role of O_2 tension

Articular cartilage is comprised of a sparsely distributed number of predominantly spherical, metabolically active chondrocyte cells comprising ~1-5% of tissue volume. They are located in lacunae contained within an ECM that is both hyperosmotic (>350 mOsm) and acidic (~pH 6.9) (Gibson et al, 2008; Lin et al, 2006). They reside in hypoxic surroundings (~5% O_2), generally respiring anaerobically through glycolysis (Lee & Urban, 1997). Chondrocytes differentiate from mesenchymal stem cells during joint development and are the only cell type in articular cartilage. Chondrocytes are responsible for the anabolic and catabolic events responsible for ECM synthesis and assembly, as well as for degradation of matrix proteins, being supplied nutrients and O_2 predominately by the synovial fluid (with a small contribution from the subchondral bone) (Gibson et al, 2008; Lin et al. 2006). Chondrocytes respond to cytokines, growth factors, and biomechanical loading (Fox et al, 2009; Milner et al, 2013). Through cytokine and growth factor stimulation, chondrocytes regulate protease and protease inhibitor production in order to finely control ECM component turnover and to cope with the functional demands of the tissue (Dijgraff et al, 1995).

Compared to other tissues, cartilage O_2 levels are comparatively low and are related to the avascular environment of cartilage. Due to the invasive nature of determining the direct oxygen concentration of mature articular cartilage, measurements of synovial fluid oxygen tensions (e.g. using an O_2 electrode directly into the joint cavity) have provided estimates of oxygen levels destined to contribute to cartilage oxygenation via diffusion. Indeed, the use of tracer isotopes confirms synovial fluid is a source of O_2, diffusing through the synovium and into the cartilage (Honner & Thompson, 1971). Through the use of hypodermic needle oxygen electrodes, the work of Ferrell & Najafipour (1992) demonstrated that oxygen tension in the synovial cavity is both blood flow and joint depth dependent being reduced if blood flow is decreased or as the probe descends deeper into the synovial cavity (towards the articular cartilage) thus confirming the presence of oxygen tension gradients within the cartilage matrix (Ferrell & Najafipour, 1992; Levick, 1995). These observations are supported by the work of

Silver (1975) who demonstrated that steep oxygen gradients exist through blocks (0.5mm thick) of articular cartilage embedded in the vascularised ear chambers of rabbits. Through the use of micro-electrodes, oxygen levels were found to be lowest in the centre of articular cartilage blocks (0.5-1% O_2) and where much more hypoxic than the vascularised connective tissue in which the articular cartilage blocks were embedded (Silver, 1975). Not surprisingly therefore, mathematical models to predict joint oxygenation expect cartilage oxygen tensions to fall proportionally to a reduction in synovial oxygenation, with the average oxygen tension of cartilage estimated to be ~5% compared to ~12% in arterial blood (Zhou et al, 2004). Other factors influencing O_2 tension within cartilage are cell density, cell distribution and oxygen consumption rate per chondrocyte cell (Zhou et al, 2004) as well as the absence or presence of joint arthropathies such as osteoarthritis (OA) and rheumatoid arthritis (RA) (Lund-Olesen, 1970).

A proportion of oxygen may be delivered from the highly vascularised subchondral bone, but to what extent is currently debatable. Some reports conclude its supply is limited and that the delivery of nutrients reaches the cartilage via the synovial fluid only (Honner & Thompson, 1971). Conversely, it has been hypothesised that the subchondral bone may be an alternative source of nutrient supply (Bashir et al, 1997), and may be responsible for up to 50% of nutrient delivery to the cartilage (Zhou et al, 2004). Thus an O_2 supply from the subchondral bone must not be discounted. Nevertheless, it is widely accepted that the concentration of O_2 from synovial fluid diffusion represents the major factor influencing the O_2 tension of articular cartilage, and thus chondrocytes (Zhou et al, 2004). It is important to recognise that oxygen levels around 5-7% are regarded as normoxic for articular chondrocytes *in situ* and that levels below these amounts should be referred to as hypoxic (Gibson et al, 2008). This is critical for the basis of the work presented in this thesis.

It is now considered that chondrocytes have conserved, adaptive mechanisms to respond to variations in oxygen levels (Pfander and Gelse, 2007). The recent interest in hypoxia inducible factors (HIF's) and their effects on induction of hypoxic response elements

(HRE's) capable of modulating chondrocyte function and survival in health and disease has supported the concept that O_2 tension is a critical parameter influencing cartilage physiology. Indeed, the material properties of cartilage as well as the deposition of extracellular matrix components within it have been observed as being highly O_2 sensitive (Fermor et al, 2007; Lafont, 2010; Henrotin et al, 2005). At oxygen levels below 5%, energy production and matrix synthesis sharply decrease (Lee & Urban, 1997; Zhou et al, 2004). Sustained low oxygen has been shown to reduce net collagen and glycosaminoglycan matrix accumulation and decrease new glycosaminoglycan synthesis almost completely (Murphy & Sambanis, 2001). Hyperoxia is also damaging and is capable of influencing chondrocyte metabolism (Mignotte et al, 1991), collagen type II and SOX9 expression (Murphy & Polack, 2004) redox status (Schneider, 2007) and mitochondrial function (Milner et al, 2007). *In vitro* reduction of O_2 tension to physiologically relevant oxygen levels (5%) promotes a chondrocytic phenotype and upregulates ECM component synthesis and deposition compared to higher, more ambient oxygen tensions (21%), highlighting the importance of this environmental parameter when studying chondrocyte function (Murphy & Polack, 2004).

During diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), O_2 tensions may be further decreased, largely due to a reduction in O_2 delivery to the tissue. Disordered capillary architecture in the joint capsule further compromises O_2 delivery in the diseased joint, with neo-vascularisation of synovial tissue only leading to complicate vessel distribution as opposed to alleviating hypoxia. Later, cartilaginous fibrillations caused by the degeneration of tissue, particularly in OA, and subsequent abnormal mechanical loading may disrupt the normal O_2 gradient as the synovial fluid infiltrates these fissures, exposing deeper layers to abnormal cytokine and growth factor levels. Osteoarthritic-mediated alterations in the synovium have also been hypothesised to cause hypoxia in the cartilage by negatively affecting oxygen transfer from the capillaries of the synovium to the synovial fluid. End stage joint disease is characterised by proliferation of synovial membrane cells, villous hypertrophy and dilation and fibrosis of venules. As cartilage is primarily dependent on O_2 delivery from the synovial fluid, such structural changes will result in compromised O_2 transfer from capillaries

across the synovium into synovial fluid and hence cartilage matrix. In addition, subchondral bone sclerosis, a feature of osteoarthritis, results in reduced oxygen delivery to the deeper cartilage layers. Indeed, in experimentally induced *in vitro* models of OA it was observed that although cellular oxygen consumption significantly increased, blood flow and diffusing capacity remained unchanged (compared to normal joints). The partial pressure difference across the synovial membrane increased >4.3 times compared to normal joint values. This is hypothesised to lead to a decrease in articular cartilage oxygen levels and enhanced cartilage hypoxia (Grimshaw & Mason, 2000; Svalagosta & Kiaer, 1989).

1.3. Chondrocytic respiratory adaptations to low oxygen tensions

Due to the low O_2 tension encountered within articular cartilage, the Embden-Myerhoff-Parnas pathway of glycolysis represents the major ATP generating metabolic pathway (~95% ATP generation) (Lee & Urban, 1997). Accordingly, ATP concentrations in articular cartilage have been shown to be relative to rates of glycolysis. As a result, mitochondrial oxidative phosphorylation is low. There are also indications that chondrocytes may not express a full complement of electron transport chain (ETC) protein complexes in the mitochondrion, but this is somewhat equivocal at present (Mignotte et al, 1991). Mitochondrial density is low in cartilage, comprising ~1-2% of total cell volume compared to 15-20% in other tissues and this is reflected by a low O_2 consumption rate (Gibson et al, 2008). Indeed it has been calculated that chondrocyte respiration rate is 26 times lower than more aerobic cells (hepatocytes, for example) (Schneider, 2007). Despite the low oxygen levels encountered *in vivo*, chondrocytes however do require and use O_2 (Lee & Urban, 1997; Gibson et al, 2008). Although chondrocytes can survive several days in anoxic conditions, glycolytic activity, matrix synthesis and ATP generation all decrease with time; highlighting the requirement for a critical level of of O_2 within cartilage (Grimshaw & Mason, 2000; Lee & Urban, 1997; Ysart & Mason, 1994). Furthermore, chondrocytes exhibit a 'negative Pasteur effect' whereby glucose uptake is inhibited by decreased O_2 levels. Indeed, a reduction in lactate production correlates closely with decreased glucose uptake by chondrocytes

(Lee & Urban, 1997). Chondrocytes also exhibit the 'Crabtree effect' where oxygen consumption is increased under conditions of glucose deprivation (Lee & Urban, 1997). Thus it appears that chondrocytes largely utilise anaerobic mechanisms for respiration but are equipped to upregulate oxidative phosphorylation in order to maintain an adequate ATP supply under limited glucose availability (Heywood et al, 2010).

1.4. pH homeostasis in articular cartilage

As well as the documented hypoxic environment, articular cartilage is also predisposed to extracellular acidification. As chondrocytes reside in a hypoxic, avascular environment and predominantly respire anaerobically, lactate is produced as a by-product of metabolism. As lactate can only be dissipated by diffusion through the matrix, the extracellular pH (pH_o) of the matrix is lowered. In addition, the abundance of large negatively charged proteoglycan molecules act to concentrate small mobile cations such as H^+, Na^+ and K^+, resulting in alterations to the extracellular ionic composition (Browning & Wilkins, 2004). Mobile anions are excluded, raising the extracellular osmolarity to ~350-450 mOsm (Tattersall et al, 2003). Additionally, as is the nature of the articulating joint, intermittent fluctuations in ionic composition are encountered during tissue loading whereby water from within the ECM is expressed, transiently increasing the concentration of fixed negative charges, resulting in streaming potentials, raised hydrostatic pressures and an influx of positive ions into the matrix, further reducing pH_o (Grodzinsky, 1983; Wilkins & Hall, 1995). Despite the crucial physiological role these features confer (such as resistance to compression), these combined effects lead to pH_o being lower than that of other tissues, with values of pH 6.9 being recorded in ECM (Browning & Wilkins, 2004; Urban, 1994).

Due to a tendency towards extracellular acidosis, chondrocytes are continually exposed to the risk of intracellular acidification. Indeed, extra and intracellular pH display a close relationship in chondrocytes (Falchuk et al, 1970; Wilkins & Hall, 1995). The seminal work by Wilkins & Hall (1995) demonstrate that chondrocyte intracellular pH (pH_i) is maintained at approximately pH 7.1 and even slight reductions in pH_o (towards acidity)

results in reductions of pH_i *in vitro*. If pH_o is significantly reduced, steep intracellular acidosis is observed. Maintenance of pH_o to values within the physiological range (pH 7.0-7.3) preserves pH_i to approximately pH 7.1 and leads to an avoidance of intracellular acidification (Wilkins & Hall, 1995).

Additionally, cartilage macromolecular components appear sensitive to changes in matrix pH, which may provide a negative feedback mechanism between the surrounding pH environment and matrix production (Wilkins & Hall, 1995). Cartilage turnover is upregulated by up to 50% at pH values between 7.1-7.2 and significantly inhibited by extracellular acidosis (by up to 75%). The observation that pH_i was unaffected by pH_o at ranges that up-regulated matrix synthesis, but decreased over the acidic ranges that inhibited matrix synthesis implies a possible modulatory effect of pH on cartilage turnover (Wilkins & Hall, 1995). In agreement with these observations, the work of Wu et al (2007) demonstrated that glycosaminoglycan production was pH sensitive in bovine articular chondrocytes cultured *in vitro*. Glycosaminoglycan production peaked when chondrocytes were cultured in pH 7.2 media and was reduced by both alkaline and acidotic conditions. Of particular significance was the observation that acidosis led to a reduction in glycosaminoglycan synthesis of up to 80% when compared to responses of chondrocytes cultured in pH 7.2 conditions, further highlighting the importance of pH_o to cartilage homeostasis.

Acidosis also occurs in joint diseases such as OA/RA (Konttinen et al, 2002) and appears to correlate with the hypoxic conditions encountered in these diseases (Lund-Olesen, 1970). The role of changes in extracellular pH in joint arthropathies however remains to be fully elucidated, despite the observation that extracellular acidosis is a clinical feature of OA, which correlates with disease severity (Levick et al, 1990). Acidosis is observed in the synovial fluid of patients with joint disease (Farr et al, 1985) and in animal models subjected to an experimental instability procedure to mimic joint disease (Kofoed, 1986). When studying pH mechanisms in chondrocytes, it is important to note that, unlike other tissues, pH_i is not controlled by HCO_3^- dependent mechanisms and evidence suggests that this buffering system plays little role in chondrocytes

(Browning & Wilkins, 2004; Simpkin et al, 2007). This appears to be due to low levels of anions such as HCO_3^- and low carbonic anhydrase activity in chondrocytes (Swietach et al, 2002). Chondrocyte pH_i is modulated primarily by the Na^+/H^+ exchanger (NHE) (Wilkins & Hall, 1992). As such, optimal functioning of the NHE is critical for cartilage and chondrocytes *in vivo*. Clearly both extracellular and intracellular pH are key environmental parameters that can govern cellular function. Typically however, *in vitro* chondrocyte investigations are carried out with bicarbonate buffered media which may be inappropriate. *In vitro* chondrocyte hypoxia has been shown to inhibit NHE activity and acid extrusion in chondrocytes (Milner et al, 2007). As extracellular pH influences intracellular pH, disease-induced acidosis of the ECM will likely lead to intracellular acidosis and altered chondrocyte functions, including ECM synthesis.

Extra- and intracellular pH has been shown to significantly modulate cellular responses in many other tissues. Although the role of pH in cartilage physiology is only beginning to become an area of interest, in other tissues (e.g. nervous tissues, cardiac tissues) it is viewed as a key environmental signal capable of modulating cell function. pH_o has been shown to be a powerful modulator of calcium activity (Zablocki et al, 2005), reactive oxygen species generation (Pekun et al, 2012; Riemann et al, 2011), intracellular pH (Riemann et al, 2011) and mitochondrial function (Zablocki et al, 2005; Pekun et al, 2012) in various cell types and it is therefore of relevance to assess the role of pH_o on chondrocyte function.

1.5. Relationships between O_2 tensions and pH in articular cartilage

The relationship between O_2 tensions and pH in cartilage physiology has only recently gained attention. From the sparse literature available however, it appears that both pH and O_2 tension can modulate chondrocyte molecular pathways and may ultimately affect cellular function (Gibson et al, 2009; Milner et al, 2006; Milner et al, 2007).

Recent findings by Das et al (2010) demonstrate that O_2 tensions and pH may act together or independently to influence chondrocyte cell function and that even minor

fluctuations in these parameters are capable of altering chondrocyte gene and protein expression *in vitro* (Das et al, 2010). This study used a novel bioreactor design to incubate human osteoarthritic chondrocytes in one of two O_2 tensions (5% and 21%) and a range of pH conditions (6.4-7.4) and observed that pH was a powerful modulator of cell function. Medium acidosis significantly reduced SOX9 gene expression, glucose consumption, lactate production and the hypoxia-regulated VEGF gene in a pH dependent manner. Culture of chondrocytes in 5% O_2 and a pH of 7.2 elicited optimal values for collagen type II, SOX9 and aggrecan gene expression. Reductions in pH_o and maintenance in 5% O_2 caused a decrease in gene expression, highlighting the important dual role that O_2 tension and pH may exert in human chondrocytes.

In addition, the work of Milner and colleagues (2006) demonstrate the significant and rapid effects that variations in O_2 tensions can have on intracellular ion homeostasis and pH_i in normal equine chondrocytes. Reductions in O_2 tension from ambient oxygen levels (20%) to a more physiological O_2 tension (5%) maintained pH_i levels to control values, however reductions from 5% O_2 (to 2.5 and 1% respectively) caused rapid and significant chondrocyte intracellular acidification within 30 minutes. This rapid reduction in pH_i was observed to be via hypoxia-induced modulation of the NHE leading to inhibition of acid extrusion across the plasma membrane. Hypoxia also compromised the ability of chondrocytes to recover from such acid loads, again through oxygen-mediated inhibition of the NHE system (Milner et al, 2006).

From these studies it appears clear that both oxygen tension and pH can significantly modulate chondrocyte cell function. The distinct and interactive effects of these environmental parameters on chondrocyte physiology however remain largely unexplored. Defining more clearly the potential interactive effects of oxygen tension and pH_o over broader ranges, such that occur under normal conditions, under states of physiological stress or in disease would more clearly define the role that these environmental parameters exert in chondrocyte physiology.

1.6. The mitochondrion: a critical organelle

Since endosymbiosis, the theory asserting that mitochondria are the result of early forms of bacteria joining preeukaryotic cells approximately 2-3 billion years ago, the mitochondrion has established and maintained its own genome, the genetic information of which crucially encodes for the synthesis and assembly of respiratory proteins necessary for mitochondrial respiration. The maintenance of a mitochondrial genome likely reflects the need for tightly regulated synthesis of proteins crucial to mitochondrial homeostasis and overall cellular function (respiration, metabolism) whilst also allowing a degree of organellular autonomy (Allen, 2002). The mitochondrion has a complex, dynamic structure that allows this organelle to fulfill the key role of energy provision via oxidative phosphorylation (Graier et al, 2007; Wallace, 2005). Research into mitochondrial function has intensified and the mitochondrion is now considered much more than just a site for energy production. Indeed, mitochondria are now accepted as contributing to many signal transduction pathways, controlling Ca^{2+} and reactive oxygen species (ROS) signaling, and in regulating cellular apoptosis (Graier et al, 2007; Granville & Gottlieb, 2002).

Structurally, mitochondria consist of an outer and inner mitochondrial membrane. The outer membrane is relatively permeable and contains transmembrane channels formed by porins, allowing movement of ions and small molecules between the mitochondria and the cytosol. The inner mitochondrial membrane (IMM) is highly folded into cristae, increasing its surface area and contains specific transporters allowing selective movement of small molecules through the membrane. Crucially however, the IMM itself (the lipid backbone) is impermeable to protons, a characteristic that is critical in adenine tri-phosphate (ATP) synthesis (Albert et al, 1998; Logan, 2006; Navarro & Boveris, 2007).

The major function of mitochondria in aerobic cells is to provide energy via synthesis of ATP through oxidative phosphorylation. Oxidative phosphorylation yields more than 15x the amount of ATP generated by anaerobic glycolysis, thus providing the major

source of energy for aerobic cellular processes (Hutteman, 2008). In aerobic respiration, the site of ATP production occurs on the inner mitochondrial membrane where the five-enzymatic multi-heteromeric complexes of the respiratory chain are embedded (complexes I, II, III, IV and V). These complexes act as reduction-oxidation (redox) pairs, catalysing electron transfer and are responsible for proton movement (Das, 2006; Rotig & Munnich, 2003; Zeviani & Di Donato, 2004). Key stages are depicted in figure **1.3**.

Electrons reduced from glycolysis and the tricarboxylic acid cycle are accepted by Complex I of the respiratory chain, which removes a hydride ion from NADH converting it to a proton and two electrons (Albert et al, 1998; Das, 2006; Rotig & Munnich, 2003). The electrons are accepted by Complex I and transferred to coenzyme Q, an electron acceptor. Similarly, Complex II transfers succinate electrons to coenzyme Q, forming ubiquinol. Complex III transfers electrons from ubiquinol to cytochrome c, another electron acceptor via two distinct sites: site Q_o and Q_i. Complex IV catalyses electron transfer from cytochrome-c to molecular O_2 and H_2O. The reduction of O_2 to H_2O provides a massive redox potential drop which maximizes the amount of free available energy for complex V and ultimate ATP synthesis and as a direct result links O_2 tension to energy generation and cellular metabolism (Hosler et al, 2006; Wilson et al, 2012). Energy released from electron transfer is used by complex I-IV to pump protons from the mitochondrial matrix to the intermembrane space, creating a transmembrane proton gradient (electrochemical gradient) that is favourable for protons to flow back into the matrix. Complex V allows the movement of protons back into the mitochondrial matrix by forming a hydrophilic proton channel across the inner mitochondrial membrane. This allows the protons to be transported down their electrochemical gradient. Complex V uses the potential energy from the electrochemical gradient to synthesise energy via condensing ADP and Pi into ATP (Gibson et al, 2008).

The formation of the electrochemical gradient results in both a pH gradient and a membrane potential across the IMM. The pH in the mitochondrial matrix is approximately one unit higher (~pH 8) than in the IMM (~pH 7), which equates to an

approximate 10-fold drop in proton concentration in the mitochondrial matrix. The membrane potential generates a gradient in charge between the matrix and the IMM whereby the matrix is negative (-180mV) compared to the IMM. The pH gradient and membrane potential work in concert to form a steep electrochemical gradient of protons. It is this gradient that makes it energetically favourable for protons to flow into the matrix from the IMM via complex V, synthesising ATP (Alberts et al, 1998) (see figure **1.3**). The mitochondrial membrane potential is a key indicator of the energisation of a cell, and depolarisation of the mitochondrial membrane potential is a hallmark of mitochondrial stress and implicit with reduced function (Skarka & Ostadal, 2002).

As chondrocyte energy synthesis is largely glycolytic, chondrocyte oxygen consumption is low which correlates with a reduction in mitochondrial density compared with other tissues (Heywood and Lee 2008) and evidence suggests that chondrocytes may lack a full complement of ETC complexes (Mignotte et al, 1991). Alternative roles for mitochondrial electron transport along an incomplete ETC have recently become a focus of interest in chondrocyte biology. Indeed, mitochondria, as in all eukaryotes, act as a key source and regulator of intracellular signalling molecules which modulate various cellular functions (Duchen, 2004). The role of mitochondrial dysfunction in OA pathogenesis has recently come to the fore. Mitochondrial density, morphology, membrane potentials and ETC protein activity are all altered during disease. As the mitochondria modulate many key biosynthetic, redox sensitive, inflammatory, catabolic and survival pathways in chondrocytes, dysfunction of this organelle would be significantly compromising and is linked to cartilage degradation and progression of disease (Blanco et al, 2011). The relationship between mitochondrial function, changes in O_2 availability and pH_o in chondrocyte physiology remain largely unexplored.

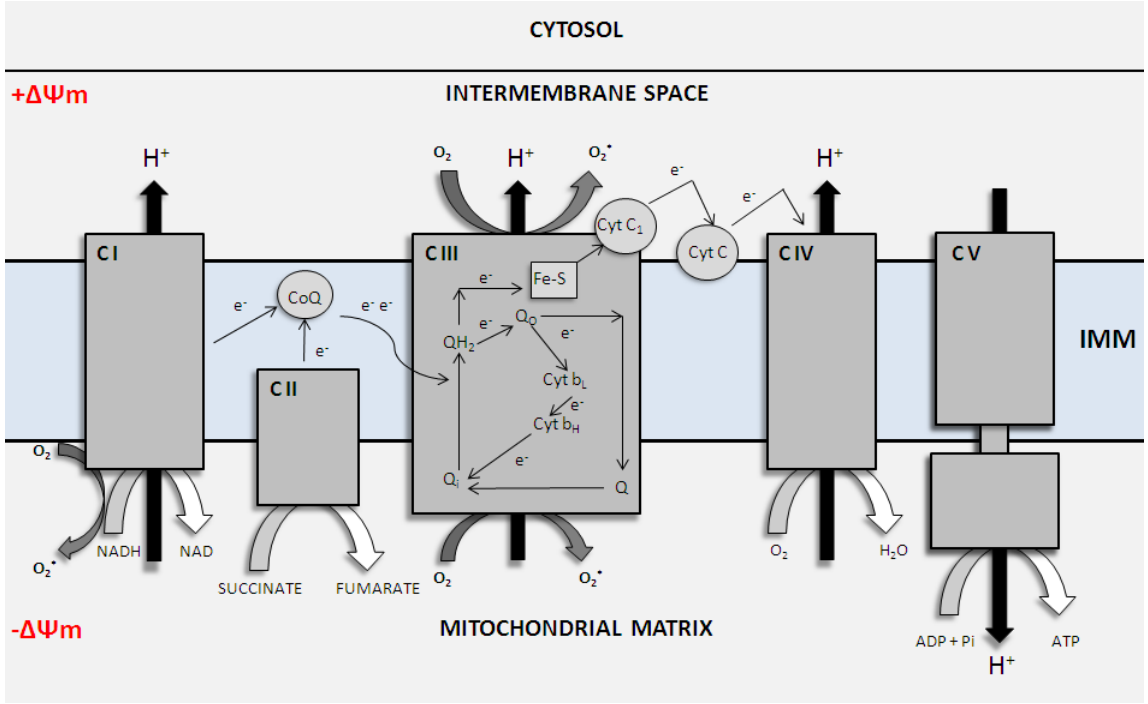


Figure 1.3. The mitochondrial Electron Transport Chain. The major function of mitochondria in aerobic cells is to provide energy through ATP synthesis via oxidative phosphorylation. Reduced substrates from glycolysis transfer electrons across a respiratory chain of ETC protein complexes (C1-CV). Each transfer represents an oxidation reduction reaction. Oxygen is the terminal electron acceptor. Some of the proteins have the crucial ability to actively pump protons across the IMM from the matrix into the intermembrane space, generating an electrochemical potential. This potential is then used to drive ATP synthesis via the final complex of the chain (CV). Crucially, the mitochondria are also the primary source of and target of reactive oxygen species (ROS) ($O_2^{\bullet}$).

1.7. Reactive oxygen species production

Free radicals (any chemical species containing an unpaired electron) and molecules that are derived from molecular oxygen (O_2) are termed reactive oxygen species (ROS). In the ground state O_2 is a relatively unreactive molecule but has the potential to become highly reactive by the gaining of electrons (reduction) (Turrens, 2003). The gaining of one electron (mono-reduction) forms superoxide anion ($O_2^{\bullet-}$) which is regarded as the most abundant oxidant produced in eukaryotes. Likewise, the gaining of two electrons forms the membrane permeable hydrogen peroxide (H_2O_2) and the gaining of three electrons forms hydroxyl radical ($^{\bullet}OH$), one of the most powerful oxidants in nature (Cash et al, 2007). Reactive nitrogen species (RNS) are free radical molecules derived from nitric oxide. NO is a well-documented intracellular messenger with important physiological roles but when overproduced, as is seen in pathologic conditions, NO can be severely deleterious due to its ability to react with ROS and form additional species, such as peroxynitrite (Bindoli et al, 2008).

The mitochondrion is considered a key source of endogenous ROS formation owing to the highly reducing environment of the ETC and abundance of redox centres (ETC complexes, iron-sulfur centres, ubisemiquinone) located therein, which act as a source of electrons that can essentially 'leak' out of the ETC and reduce O_2 under certain conditions (Turrens, 2003). This process is thermodynamically favourable (Turrens, 2003) and approximately 0.2-2% of total O_2 consumed during electron transport is incompletely reduced spontaneously to form $O_2^{\bullet-}$ under quiescent conditions (see figure **1.3**) (St-Pierre et al, 2002). $O_2^{\bullet-}$ is the primary ROS produced by mitochondria and acts as the precursor for many secondary ROS and RNS (such as H_2O_2, $^{\bullet}OH$ and peroxynitrite (the result of $O_2^{\bullet-}$ reacting with nitric oxide)) being either vectorially released into the mitochondrial matrix (~80%) or the intermembrane space (~20%) (Navarro & Boveris, 2007; Turrens, 2003; Valko et al, 2007). This is more likely to occur when the mitochondrial membrane potential is high and thus electron transfer in the ETC is increased and the potential for electron slippage to O_2 is enhanced. In this sense, mitochondrial function, O_2 and ROS/RNS production are inextricably linked (Gibson, et al 2008).

ROS/RNS are important molecules participating in a plethora of signalling cascades as well as being implicated in many damaging and degenerative processes if overproduced. These dual roles are well documented and ROS/RNS should not be seen solely as deleterious free radicals but also as a developed response capable of controlling many mechanisms accountable for various physiological functions (Valka et al, 2007).

Since the concentration of oxygen can influence mitochondrial membrane potential, oxygen levels are a key determinant of potential ROS formation. Observed differences of tissue responses to oxygen tension and subsequent ROS generation appear to be dependent on factors such as the O_2 requirements of the tissue in question, the source of ROS, the level of hypoxia and presence and pathophysiology of disease (Boveris & Chance, 1973; Costa et al, 1993; Chandel, 2000; Duranteau et al 1998; Milner et al, 2007; Solaini et al, 2010). Many studies in aerobic tissues have shown increased generation of ROS in hypoxic conditions (Chandel, 2000; Solaini et al, 2010). Duranteau et al (1998) observed an increase in mitochondrial ROS generation as the concentration of O_2 decreased from 21%, to 5, 3 and 1% in contracting cardiomyocytes. Reductions in O_2 tension increased ROS production in an O_2 dependent manner which negatively affected cardiomyocyte contractility.

Conversely, anaerobic tissues such as cartilage may display different responses. The work of Milner et al (2007) shows that ROS generation significantly falls as the level of oxygen availability is reduced from physiological concentrations (5%) to hypoxic levels (1%) in normal equine articular chondrocytes. This hypoxia induced reduction in ROS levels paralleled inhibition of NHE activity resulting in significant intracellular chondrocyte acidification. The reduction of ROS generation in hypoxia appeared to be due to a reduction in mitochondrial membrane potential, which is implicit with a reduction in electron transfer in the ETC, thereby reducing the flux of electrons available for partial reduction of O_2 to form ROS (Milner et al, 2007). These findings demonstrate an oxygen sensitive link between mitochondrially derived ROS generation and chondrocyte cellular activities such as intracellular pH

regulation. As a result it is interesting to speculate the potential role that this link may play in redox signalling in normal and abnormal (disease) joint physiology.

Under normal conditions, to maintain cellular homeostasis and redox balance, the endogenous formation of ROS/RNS must be counteracted by the cells intricate antioxidant defences, namely catalase, superoxide dismutase and glutathione peroxidase (Gao et al, 2008). In normal resting cells, the antioxidant systems are efficient enough to almost completely abrogate the effect of ROS generation (Gao et al, 2008).

Despite recent evidence highlighting the participation of ROS/RNS as intracellular messengers and potential oxygen 'sensors' through interactions with hypoxic inducible factors (Guzy et al, 2005), ROS are also implicated in most if not all major diseases including many cancers, cardiovascular diseases and neurological diseases (Brieger et al, 2012). The deleterious processes resulting from an imbalance between ROS/RNS overproduction and an inadequate capability of antioxidant systems to detoxify ROS is termed oxidative stress and is well documented in many tissues. In pathological conditions, oxidative stress can irreversibly modify all components of the DNA molecule as well as permanently and indiscriminately alter proteins and lipid structures (Addabbo et al, 2009; Droge, 2002).

Overproduction of ROS and RNS is implicated in the pathology of joint diseases. Excessive ROS has been shown to inhibit matrix anabolism through inhibition of glycolytic enzymes (Henrotin et al, 2003) or mitochondrial ETC complex inhibition, both of which would lead to reduced ATP formation (Li et al, 2012). Increased ROS levels and reduced antioxidant levels are observed in OA patients compared with individuals without OA which correlates with increased DNA damage (Altindag et al, 2007) and oxidative stress (Regan et al, 2008). Overproduction of ROS is also implicated in potentiation of chondrocyte apoptosis (Del-Carlo and Loeser, 2002), induction of matrix metalloproteases, inhibition of cartilage matrix synthesis and upregulation of pro-inflammatory cytokines, all of which are heavily implicated in irreversible cartilage degradation. The relationship between ROS and NO (leading to peroxynitrite formation) is well documented and contributes to OA pathogenesis,

being accentuated by pro-inflammatory cytokine stimulation (Afonso et al, 2007). Markers of protein oxidation such as nitrotyrosine (the result of protein tyrosine residues reacting with peroxynitrite) are observed in OA (Afonso et al, 2007) and overproduction of NO induces cartilage catabolism, increasing proteoglycan breakdown (Tomita et al, 2001). As the majority of ROS are produced in the mitochondria, mitochondrial proteins, mitochondrial DNA (which lack protection from histones) and mitochondrial membranes are especially open to damage by ROS. ROS can modulate many mitochondrial pathways which can significantly contribute to mitochondrial dysfunction. Mitochondrial ROS overproduction impairs the ability of this organelle to carry out a wide array of metabolic functions and prolonged oxidative stress can alter membrane permeability and induce the release of intermembrane proteins such as cytochrome *c* to ultimately activate cellular apoptosis. Additionally, ROS induced mitochondrial outer membrane permeabilisation (MOMP) and activation of the permeability transition pore (PTP) (on the inner mitochondrial membrane) alters membrane integrity and protein transport, ultimately leading to cell death. ROS damage leading to mitochondrial dysfunction is therefore a key feature of many pathologies (Murphy et al, 2009) (see figure **1.4**). As such, the role of ROS/RNS overproduction, cellular antioxidant depletion and mitochondrial dysfunction is a growing area of research in the pathogenesis of many diseases (reviewed in Brieger et al, 2012; Kirkinezos et al, 2001; Lenaz et al 2001; Valko et al 2007) including those involving structures of the articular joint (Henrotin et al, 2003; Henrotin et al, 2005; Li et al, 2012). The role of oxygen tension and pH_o in these processes is as yet, unidentified.

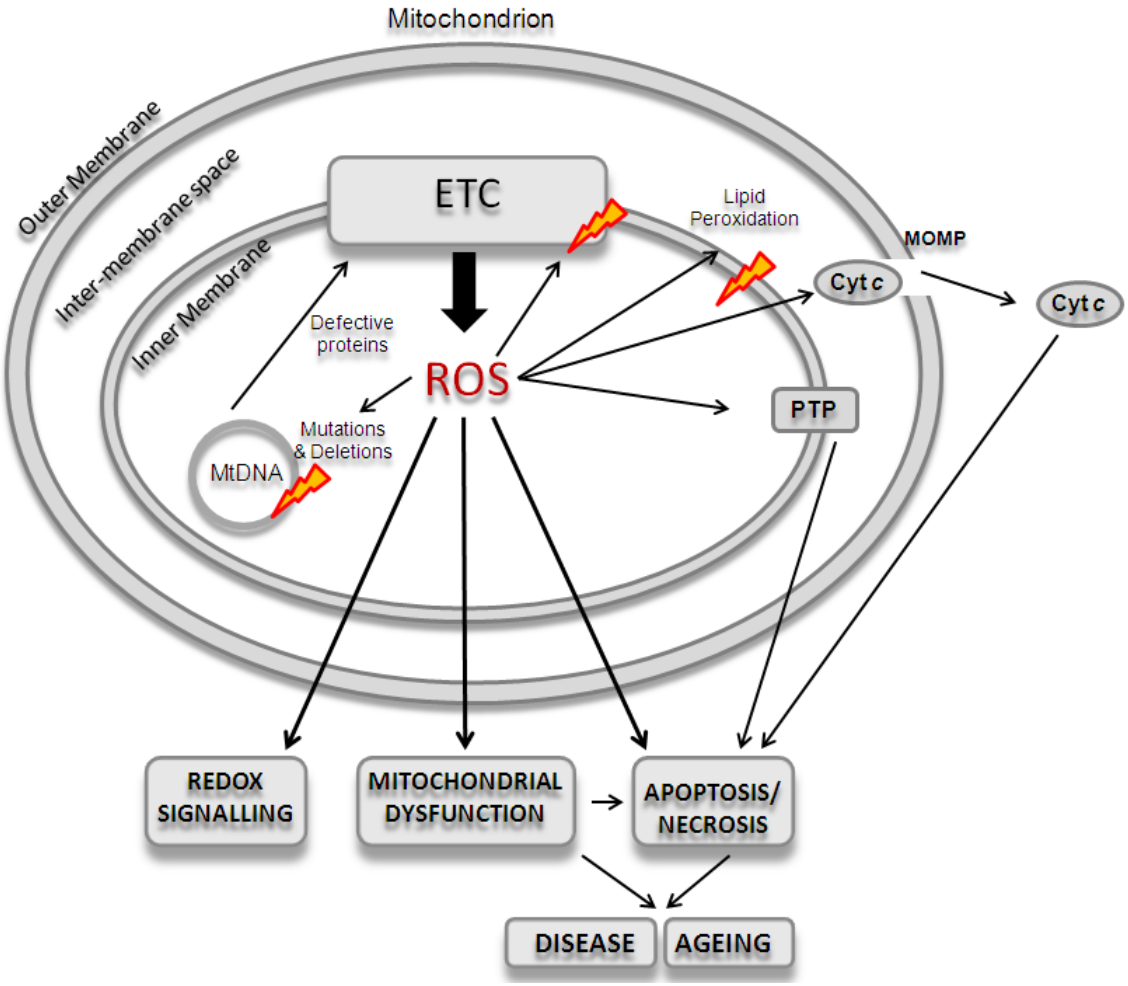


Figure 1.4. Effects of mitochondrially derived ROS on mitochondrial homeostasis. The mitochondrial ETC is a key generator of ROS formation in chondrocytes. Chronic ROS overproduction, as is seen in joint disease, leads to altered redox signalling and redox imbalance which are associated with chondrocyte apoptosis, lipid peroxidation, increased cytokine activity, ECM degradation and altered energy synthesis. Perturbations in normal mitochondrial homeostasis can alter mitochondrial DNA, protein formation and integrity of membranes. As mitochondria are regulators of apoptosis, ROS induced mitochondrial dysfunction can lead to increased cell death through cytochrome *c* release (cyt *c*) or opening of the mitochondrial outer membrane pore (MOMP). As a result, mitochondrial dysfunction and ROS overproduction is linked to OA and aging processes.

1.8. Antioxidant systems in cartilage

As chondrocytes are long lived cells and cartilage does not possess the mechanisms by which to replace damaged or dead chondrocytes, a sustained increase in oxidative stress would be significantly compromising to cartilage integrity. As such, reductions in cellular antioxidant capacity may have deleterious effects on the ECM of articular cartilage implicit with ageing (Loeser et al, 2009; Sredzinska et al, 2009) and diseases of the joint (Ballatori et al, 2009). Antioxidant enzymes are distributed ubiquitously throughout the body and include but are not limited to superoxide dismutases and glutathione peroxidase which work in concert under normal conditions to detoxify $O_2^{\bullet-}$ and H_2O_2 respectively (see figure **1.5**) (Afonso et al, 2007).

Superoxide dismutases

Superoxide dismutases (SODs) are metalloenzymes that are widely distributed in eukaryotic cells and constitute a family of proteins that dismutate $O_2^{\bullet-}$ in a two-step process to produce H_2O_2 (figure **1.5**) (Johnson & Giulivi, 2005). As $O_2^{\bullet-}$ is the primary ROS generated from molecular O_2 and acts as the precursor for many other ROS and RNS, the role that SODs play in prevention of oxidative stress is central. Three SOD isoforms currently exist; SOD1, SOD2 and SOD3 with each isoform being defined by the metal ion they bind and/or their cellular localization, SOD1 (Cu-ZnSOD; cytosol and the mitochondrial intermembrane space), SOD2 (MnSOD; exclusively the mitochondria), SOD3 (ECSOD; extracellular compartment) (Afonso et al, 2007; Scott et al, 2012; Tsunoda et al 2013). As SOD can only dismutate $O_2^{\bullet-}$ to H_2O_2, the actions of SOD must be in concert with H_2O_2 scavenging antioxidant systems (primarily glutathione, see below). If H_2O_2 levels increase in the presence of Fe^{3+}, this favours its conversion to the powerful hydroxyl radical, via the Fenton reaction. As such a coordinated, efficient global cellular antioxidant system is needed to protect from the adverse effects of prolonged ROS overproduction (Johnson & Giulivi, 2005).

The use of *Drosophila* systems have been elegantly used as models of oxidative stress to demonstrate the central role that SODs play in cellular homeostasis and lifespan. Indeed overexpression of SOD1 has been shown to induce resistance to oxidative stress by increasing the metabolism of $O_2^{\bullet-}$, ultimately increasing *Drosophila* lifespan by up to 40% compared to control (Parkes et al, 1998). Inversely, *Drosophila* deficient in both SOD1 and SOD2 undergo premature death by ~20 days compared to control (~50 days) (Wicks et al, 2009). Additionally, the lifespan of *Drosophila* was observed as being significantly oxygen-sensitive. Maintenance of SOD-1 and SOD-2 deficient flies in hypoxic (5% O_2) conditions mirrored control values and completely abrogated the premature death observed in flies maintained in 21% O_2 conditions. Furthermore, switching environmental conditions from ambient oxygen tensions (21%) to hypoxia (5%) when mortality hit 50% of the population significantly arrested mortality and prolonged survival rates. Although ROS were not directly measured in this study, the observed discrepancies in lifespan as a result of exposure to different oxygen environments were attributed to a hypoxia-induced reduction in mitochondrial $O_2^{\bullet-}$ generation and support the free-radical theory of ageing (Wicks et al, 2009).

In terms of cartilage physiology, all SOD isoforms are highly expressed in normal human cartilage implying this tissue has an active ROS producing capacity in need of continual ROS scavenging systems to maintain normal function. During joint diseases such as OA, all SOD isoforms are significantly reduced which correlates with an increase in $O_2^{\bullet-}$ generation. Down regulation of SOD isoforms in OA chondrocytes appears to precede OA lesions, implicating reduced SOD levels as a contributing factor in early stage OA pathogenesis. Additionally, SOD2 depletion leads to increased mitochondrial DNA mutations and subsequent mitochondrial dysfunction (Scott et al, 2012).

Glutathione

Glutathione, a thiol containing compound, in its reduced form (GSH), is regarded as a key antioxidant molecule inside the cell, scavenging H_2O_2 formed as a result of $O_2^{\bullet-}$ dismutation by SOD (Gao et al, 2008). GSH is synthesised in the cytosol by

both glutamate-cysteine ligase and GSH synthetase. Glutamate-cysteine ligase acts as the rate limiting enzyme in GSH synthesis as it possesses a modifier subunit that can mediate the activity of the enzyme by altering the affinity of its catalytic subunit for inhibitors and substrates (Giordano et al, 2011). Approximately 90% of glutathione resides in its reduced form (GSH) and acts as an electron donor, reducing H_2O_2 (in the presence of the key enzyme; glutathione peroxidase). As a result, GSH becomes oxidized into glutathione disulfide (GSSG). A high GSH:GSSG ratio is important for protection against oxidative stress and this ratio is used as a marker of intracellular redox balance (Blokhina & Fagerstedt, 2010). The normal ratio of GSH:GSSG in mitochondria has been observed as high as 18:1, and fluctuations in this ratio can have marked intracellular effects (Giordano et al, 2011). From the cellular pool of GSH, only small amounts are sequestered into the mitochondria, being transported from cytosolic pools or being regenerated from GSSG, catalysed by glutathione reductase (GR) (figure **1.5**). This process is fundamental in antioxidant biology and both the reduced and oxidized forms of glutathione allow for fine tuning of the cellular redox environment through GSH stress signaling (Gao, et al, 2008).

Since GSH is a crucial player in redox balance, disturbances in GSH homeostasis have been implicated in the aetiology of numerous diseases, including many cancers, neurodegenerative, inflammatory, cardiovascular, immune, metabolic and age-related diseases. Appropriate levels of cellular GSH appear crucial for survival (Ballatori et al, 2009). Human articular chondrocytes treated with GSH inhibitors show reduced GSH levels, a more oxidised thiol redox status and a significant increase in cell death under conditions of oxidative stress. In contrast, inducing a reduced thiol redox status increases GSH levels and leads to protection against oxidative stress induced by exogenous $O_2^{\bullet-}$ addition. A decrease in the GSH:GSSG ratio in older donors is evident and implies an increase in oxidative stress at baseline for these older patients. Accordingly, older chondrocytes (from patients >50 years) show reduced survival rates compared to younger chondrocytes (from patients18-49 years) when exposed to oxidative stress. As OA is age-related and chondrocyte apoptosis is evident in some (but not all) studies, the correlation between a reduced GSH:GSSG ratio and increased cell death in older chondrocytes lends evidence to

the hypothesis that reduced GSH levels may be a clinical feature of OA leading to oxidant-mediated cell death (Del-Carlo & Loeser, 2003).

The importance of oxygen and pH signaling on mitochondrial function is only beginning to be recognised. The distinct and interactive relationship between these parameters and ROS/RNS and antioxidant status in chondrocytes also remains to be elucidated. Additionally, in an attempt to replenish antioxidant enzymes and counter disease-associated oxidative stress, ROS regulation provides a promising avenue of research for disease modification

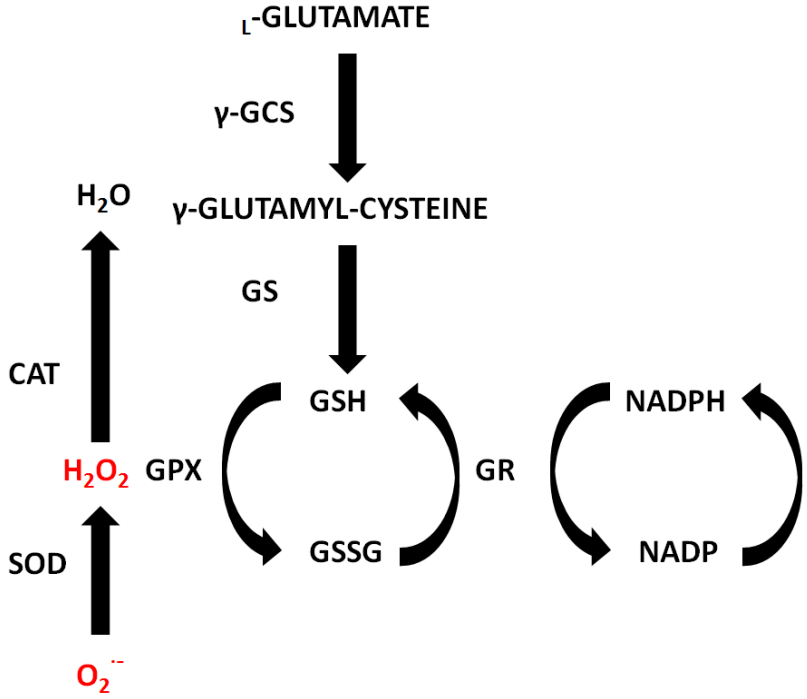


Figure 1.5. Antioxidant systems for the effective removal of ROS. The three antioxidant systems in cartilage are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). SOD dismutates the superoxide radical to oxygen and H_2O_2. Catalase and GPx catalyse the conversion of H_2O_2 into water. A high GSH:GSSG ratio is important for protection against oxidative stress and this ratio is used as a marker of intracellular redox balance. Fluctuations in the balance between antioxidant capacities and ROS formation can have marked effects on cellular function. In pathological conditions, such that are seen in joint disease, overproduction of ROS may be evident with an insufficient capacity to remove the oxidants. Under these circumstances the potentially damaging effects of ROS may predominate, leading to a decrease in cell function and integrity.

1.9. Hypothesis

Articular chondrocytes live in a unique environment in cartilage characterised by low oxygen conditions and acidosis. Despite low consumption, articular chondrocytes still have a requirement for oxygen and current findings demonstrate a direct link between oxygen availability and ROS production in chondrocytes. Furthermore, a role for mitochondrially-derived ROS in chondrocyte cell signalling has been demonstrated (Milner et al 2007) and research into the modulatory role of appropriate and inappropriate oxygen tensions in cartilage biology is intensifying. The role that extracellular pH plays in chondrocyte function is relatively unknown despite the wealth of literature highlighting the crucial role this environmental parameter exerts in other tissues (Pekun et al, 2012; Riemann et al, 2011; Zablocki et al, 2005). How chondrocyte mitochondrial function and redox balance is altered in response to varying environmental parameters such as oxygen and pH is currently unknown. Elucidating the role of mitochondrial function and redox signalling in (patho) physiological conditions is vital to improving our fundamental understanding of chondrocyte physiology in relation to joint health and disease. Although the effect of exogenous antioxidant addition on redox balance is recognized, the effects of these agents on mitochondrial function are less well understood. Additionally, the interacting effects that changes in oxygen tension and extracellular pH may have on the efficacy of antioxidant treatment are as yet unidentified.

In this project we will address the hypothesis that oxygen tension and extracellular pH are important modulators of mitochondrial physiology and chondrocyte redox balance and cellular function. We also aim to identify the ability of antioxidant compounds to modify mitochondrial function and cellular redox balance under these conditions.

1.10. Aims

The aims of this project are fourfold:

(1) To analyse cellular responses to changes in oxygen tension, extracellular pH and cytokine stimulation over time (96 hours) in articular chondrocytes cultured in a three dimensional alginate model. Environmental conditions will be designed to reflect both physiological and pathophysiological conditions experienced by articular cartilage

(2) To investigate the distinct and interactive effects of changes to oxygen tension, extracellular pH and cytokine stimulation on chondrocyte mitochondrial function and redox balance

(3) To investigate the ability of exogenous antioxidant addition to manipulate mitochondrial function and redox balance under these environmental parameters

(4) To investigate the mechanisms involved in how oxygen tension, extracellular pH and antioxidant compounds modulate chondrocyte function

Chapter 2

Materials and Methods

2.1 Materials

Dulbecco's modified Eagle's medium (1g/L glucose), the fluorescent probe BCECF-AM and the chemicals nigericin, interleukin-1β, Trypan Blue, dimethyl sulfoxide (DMSO), and sodium dithionite were obtained from Sigma-Aldrich, Dorset, UK. Also from Sigma-Aldrich were the antioxidant compounds resveratrol and N-acetyl-cysteine and the Nitrite/Nitrate Assay Kit (colorimetric). Fluorescent probes (DCFH-DA, JC-1) were obtained from Invitrogen, Paisley, UK. The GSH:GSSG-Glo™ Assay was supplied by Promega, Southampton, UK. The MMP13 Human ELISA kit was obtained from Abcam, Cambridge, UK. Enzylight™ ATP Assay kits were purchased from Gentaur Ltd, London, UK. All Western blotting materials were purchased from Invitrogen, Life Technologies Corporation, Paisley, UK. A full list of antibodies used in Western blotting is given in table **2.1**. The BCA protein quantification assay and Novex® pre-stained lane marker were purchased from Thermo Fisher Scientific, Pierce Protein Biology products, Loughborough, UK. The Cytotoxicity Detection Kit ^{Plus} and Protease inhibitor cocktail tablets (Complete Mini) were purchased from Roche Applied Sciences, Sussex, UK. The Western Lightning-Plus ECL chemiluminescence Detection Kit was obtained from PerkinElmer, Buckinghamshire, UK. Finally, Vectashield mounting buffer was purchased from Vector laboratories, Peterborough, UK.

In this thesis, the term 'normal growth media/culture media' refers to bicarbonate free DMEM (D2902) titrated to pH 7.2 (buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)). For this, growth media powder was dissolved in one litre of ddH_2O using a magnetic stirrer. Media titrated to pH 7.2 was used for all digestion and cell maintenance protocols and experimental incubations. For media requiring a pre-titrated pH of 6.2, DMEM was buffered with 10 mM piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES). Growth medias were pre-titrated to pH 7.2 or pH 6.2 using NaOH or HCl. All media was sterile filtered using one litre sterile filter units (Thermo Fisher Scientific, Nalgene, and Loughborough, UK) and a dry vacuum pump (2511, Welch, Hampshire, UK). Sterile media was supplemented with sterile foetal calf serum (10%), ascorbate-2-phosphate (25µg/ml) and antibiotics (100 units/ml penicillin, 100mg/ml streptomycin and 500ng/ml

Amphotericin B). For anoxic media, pH 7.2 or pH 6.2 titrated media were supplemented with 1.5 mM sterile sodium dithionite to scavenge residual oxygen. Sodium dithionite supplemented media was then maintained in a 2% O_2 environment for 48 hours. All medias were pre equilibrated to their respective O_2 tensions for 48 hours prior to experimental incubations. DMSO was the solvent of choice for the fluorescent probes BCECF-AM, JC-1and DCFH-DA. The antioxidant compounds N-acetyl-cysteine (2mM) and resveratrol (10µM) were reconstituted in ddH_2O and DMSO respectively.

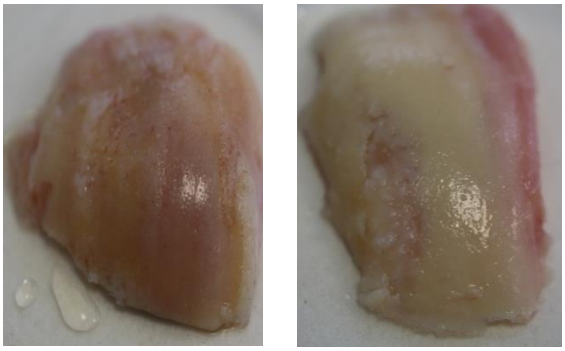
2.2 Cell culture

2.2.1. Isolation of primary articular chondrocytes

Human osteoarthritic articular cartilage (HOAC) was obtained from knee joints removed by total-knee arthroplasty (figure **2.1A**). Approval and informed consent was obtained in accordance with the Cheshire Research Ethics Committee. Equine hyaline articular cartilage (EQHAC) was obtained from the grossly normal metacarpophalangeal joints of skeletally mature equine forelimbs obtained from an abattoir or that were euthanased for clinical reasons not concerning these investigations. Approval and consent was obtained in accordance with local ethics committee guidelines. All equine cartilage was macroscopically graded using a modified version of the macroscopic grading system put forth by Little et al (2010) (figure **2.1B**), and were all graded as normal (score=0), with no signs of surface roughening, fibrillations or erosions, and therefore no signs of OA (figure **2.1C**).

HOAC and EQHAC were processed to yield chondrocytes using the protocol detailed by Tew et al (2008). For human tissue, under aseptic conditions, full thickness, macroscopically intact cartilage was dissected from the subchondral bone of the tibial plateau and medial and lateral femoral condyles using a sterile scalpel blade and mixed randomly. Similarly, for normal equine cartilage, full thickness, macroscopically intact cartilage was aseptically dissected from all surfaces of the metacarpophalangeal joint (metacarpal, phalangeal and sesamoid surfaces of phalanx 1) and mixed randomly. Dissected cartilage was entered into growth medium. Cartilage pieces were finely diced and digested overnight in growth medium plus 0.08% bacterial collagenase type II in a 37˚C, 21% O_2 humidified atmosphere under continuous agitation. After complete digestion, the cell suspension was strained using sterile cell strainers and centrifuged, yielding primary isolated chondrocytes.

A



B

Gross articular damage – score each area separately		Score
Assessment of central cartilage of	Medial tibial condyle Lateral tibial condyle MFC LFC	
Normal		0
Surface roughening		1
Fibrillation and fissures		2
Small erosions down to subchondral bone (<5 mm diameter)		3
Larger erosions down to subchondral bone (>5 mm diameter)		4
Add to give lesion score		0–16

C

Figure 2.1. Gross appearance of articular joints. A: Gross appearance of human osteoarthritic articular cartilage (femoral condyles). Note the appearance of erosion to expose the subchondral bone (left panel) and surface roughening and fibrillated cartilage (right panel). **B**: Reference guide for macroscopic grading of cartilage as put forth by Little et al (2010). **C**: Gross appearance of normal equine articular cartilage (distal aspect of the third equine metacarpal bone). Note the absence of surface roughening, fibrillations and fissures and erosion, score=0.

2.2.2. Monolayer expansion of primary articular chondrocytes

Isolated chondrocytes were washed twice in sterile 1X phosphate buffered saline (PBS). For monolayer culture, chondrocytes were maintained and expanded in sterile Cellstar T175[2] tissue culture flasks. Cells were grown and maintained in 25 mls of DMEM (HEPES buffered, pH 7.2), containing 10% foetal calf serum, ascorbate-2-phosphate (25µg/ml) and antibiotics (100 units/ml penicillin, 100mg/ml streptomycin and 500ng/ml Amphotericin B) at a seeding density of $2x10^4$cells/cm^2 and incubated at 37˚C in a 90% N_2 and 5% CO_2 humidified atmosphere (5% O_2 environment). Growth media was refreshed every 48 hours.

Cells were passaged on achievement of 70-90% confluence. Cell count and growth assessment was determined by haemocytometry. For maintenance, cells were split in a 1:2 ratio. To recover the chondrocytic phenotype, monolayer cultured cells were trypsinised and cultured in a three-dimensional alginate culture system by passage 3 (Tew et al, 2008).

2.2.3. Determining cell number

For appropriate cell seeding densities, cells were centrifuged at 394 rcf for 4 minutes to yield a cell pellet. The cell pellet was resuspended in 1 ml of culture media and 10 µl of the cell suspension was pipetted into a haemocytometer (Hawksley, Sussex, UK). Cells were counted manually using an inverted microscope (Nikon Eclipse TS-100, Surrey, UK).

2.2.4. Measurement of cell viability

Cell viability was assessed using the Trypan blue exclusion method. Cell viability was always >90% when using human or equine tissue. Monolayer cultured cells were trypsinised and washed in sterile 1X PBS. Alginate beads were depolymerised as described later and resuspended in equal volumes of sterile 1X PBS. A 40µl aliquot from each condition was mixed with 10µl of Trypan Blue solution, loaded onto a dual chamber haemocytometer and the total number of cells and total number

of blue stained cells were counted manually. The trypan blue exclusion method is based on the principle that live cells possess intact cell membranes and can therefore exclude the trypan blue dye whereas damaged and non-viable cells cannot. Percentage cell viability was calculated using the equation:

% viable cells = live cell count/total cells * 100

2.2.5. Culture of monolayer expanded chondrocytes in alginate beads

Chondrocytes entrapped in a three-dimensional alginate culture system, at high density, have been shown to promote the synthesis of markers of a chondrocyte phenotype. Furthermore, non-chondrogenic markers of dedifferentiation are decreased. Alginate is a linear polysaccharide which forms a gel in the presence of calcium and can be easily solubilised by chelating agents and centrifugation, releasing the chondrocytes after a period of culture. Alginate bead culture returns chondrocytes to their three dimensional scaffold, replicating the structure of the *in vivo* cartilaginous matrix (De Ceunick et al, 2004; Hauselmann et al, 1994).

Confluent HOAC and EQHAC were twice washed in Hank's Balanced Salt Solution (HBSS) and trypsinised. Resuspension in growth media preceded cell growth assessment by haemocytometry. Cells were centrifuged (394 rcf for 4 minutes), washed (5mls 1X PBS) and resuspended in a 1.2% sterile alginate solution (in 150mM NaCl) to give a cell density of $4x10^6$ cell/ml.

The alginate/cell solution was aspirated into a 20ml sterile syringe and capped with a 25-gauge sterile needle and the alginate/cell suspension was released dropwise into sterile 15cm Petri dishes containing 40ml calcium chloride (102mM). After instantaneous gelation, the beads were left to polymerise for 10 minutes. The calcium chloride solution was removed and two washes with 25ml of sodium chloride (150mM) preceded addition of 100mls growth medium to Petri dishes. Beads were then cultured in a 5% O_2 humidified environment (90% N_2 and 5% CO_2) for 14 days. Media was replenished every 48 hours.

2.2.6. Culture of primary chondrocytes in alginate beads

Primary isolated HOAC and EQHAC were not passaged or expanded in monolayer culture. Isolated cells were washed twice in sterile 1X PBS and cell viability was determined by Trypan blue exclusion. Cells were then directly seeded and cultured in alginate at a cell density of $4x10^6$cells/ml (as described) and cultured for 14 days in a 5% O_2 tension (humidified atmosphere). Media was replenished every 48 hours.

2.2.7. Recovery of chondrocyte cells from alginate encapsulation

To release cells from their alginate matrix, beads were depolymerised in 105mM sodium citrate solution (containing 0.9% 150mM NaCl, pH 6.0). Alginate beads were incubated at 37˚C in a humidified shaking incubator for 5 minutes until complete depolymerisation of the alginate had occurred. The remaining cell solution was centrifuged at 394 rcf for 4 minutes and the supernatant was discarded. The resulting cell pellet was washed twice in sterile 1X PBS ready for analysis.

2.3. Western Blotting

For the detection of specific proteins, Western blotting was carried out on cell lysates. Alginate beads prior to (t=0), or after experimental incubations (t=48), were depolymerised and centrifuged at 394 rcf and the supernatant was discarded. Cell pellets were washed twice in equal volumes of sterile 1X PBS and resuspended in SDS lysis buffer (containing a protease inhibitor cocktail) to lyse the cells. Each cell lysate was sonicated and cell lysates were frozen and stored at -20°C until western blot analysis.

2.3.1. Pierce BCA protein assay protocol

The Pierce bicinchoninic acid (BCA) protein assay kit was used for the colorimetric detection and quantification of total protein samples. Bovine serum albumin (BSA) standards of known protein concentration (0-2000 µg/ml) were prepared according

to manufacturer's instructions. The necessary working reagent (WR) volume was calculated using the equation:

Total volume of WR required = (# of standards + # of unknown samples) * (# of replicates) * (volume of WR needed)

25 µl of protein standards and unknown protein samples were entered in triplicate into a 96-well plate. 200 µl of BCA WR was added to each unknown protein sample and BSA standard solution. The 96-well plate was mixed on a plate shaker for 30 seconds, and incubated at 37˚C for 30 minutes. After incubation, absorbance was measured at a wavelength of 570nm on a plate reader (Multiskan FC, Thermo Scientific, Loughborough, UK). A standard curve was created from the BSA standards. Using the linear regression equation from the standard curve, the determination of protein concentrations from the unknown protein samples was calculated (figure **2.2**).

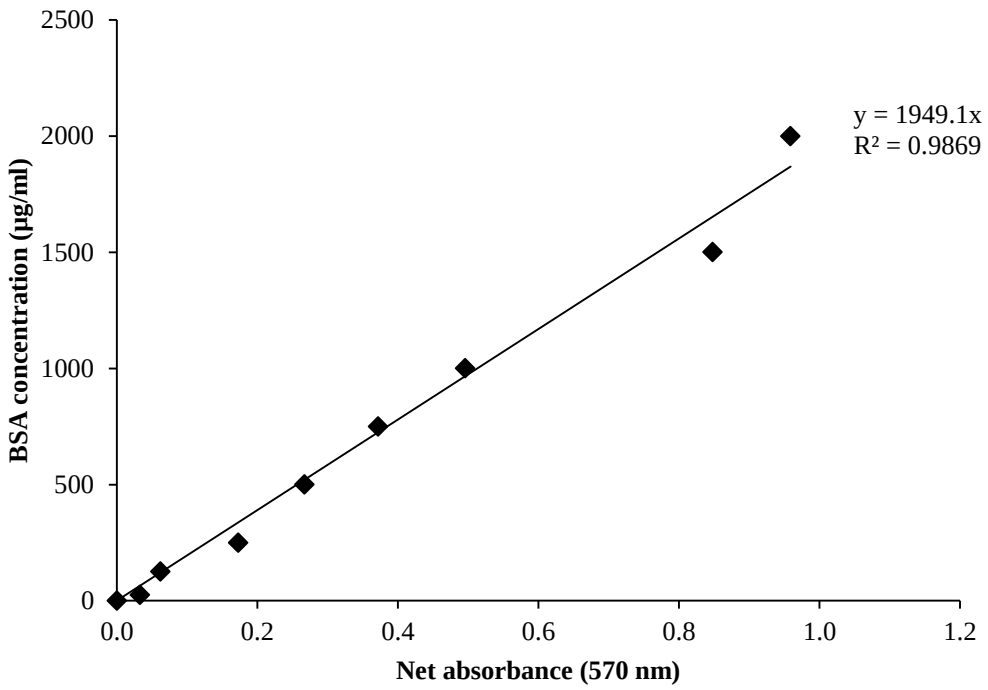


Figure 2.2. Typical standard curve for BSA used for quantification of protein from unknown cell lysates. Final concentrations of BSA ranging from 0-2000 µg/ml were entered into wells of a 96 well plate (25 µl) in triplicate and 200µl of working reagent was added into each well. Absorbance was immediately read on a spectrophotometer at an absorbance of 570nm.

2.3.2. Sodium Dodecyl sulphate-polyacrilamide gel electrophoresis (SDS PAGE)

Protein concentrations of unknown samples were calculated (see above) and 10µg of protein from each sample was mixed with 5µl of non-reducing lane marker and 2µl of DTT (for reducing conditions). Protein samples were placed in a heated rack at 80˚C for 10 minutes to denature samples.

A NuPage® 4-12% Bis-Tris Gel cassette was rinsed in distilled water. The spacer comb and adhesive strip from the foot of the gel was removed and the cassette was inserted into a gel tank. Gel cassettes were locked in position using a gel tension wedge. The upper and lower buffer chambers of the gel tank were filled with 1X running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7) ensuring all wells of the gel were submerged. After sufficient heat treatment, protein samples were centrifuged at 394 rcf and each sample was individually loaded into separate wells of the running gel using loose gel tips. Ten microlitres of Novex pre stained protein standard was entered into a final well to visually confirm molecular weights. The gel tank lid was attached and connected to an electrical source (Powerpac 300, Biorad, Hertfordshire, UK) and subjected to a constant voltage (200V) for 35 minutes (electrophoresis) to separate proteins by molecular weight.

2.3.3. Protein transfer

Electrophoresed proteins were transferred onto a nitrocellulose membrane. Sponge blotting pads were soaked in transfer buffer (25 mM Bicine, 25 mM Tris Base, 0.05mM Chlorobutanol, 1 mM EDTA, pH 7.2). Pure nitrocellulose transfer membrane and 2 filter papers were cut to the same dimensions as the running gel. The nitrocellulose membrane was soaked in transfer buffer for 15 minutes. After electrophoresis, the gel cassette was opened and the gel was removed. A gel/blot sandwich was created by placing one piece of filter paper (pre-soaked in transfer buffer) on the underside of the running gel. The pre-soaked nitrocellulose membrane was placed on the blotted side of the gel and the second piece of pre-soaked filter paper was placed on top of the nitrocellulose membrane. All air bubbles were smoothed out. Three sponge blotting pads pre-soaked in transfer buffer were placed

in a blot module (Xcell II, Invitrogen, Paisley, UK) cathode and the gel/blot sandwich was placed on top of the sponge pads. Finally, the remaining pre-soaked sponge blotting pads were placed on top of the gel/blot sandwich and the anode was placed on top of the pads. This orientation ensured the nitrocellulose membrane faced the positively charged anode.

The blot module was placed in the gel tank and fixed in place with the gel tension wedge. The blot module was filled with transfer buffer to prevent overheating and connected to an electrical source and subjected to a constant power supply held at 30V for 90 minutes. After this time the nitrocellulose membrane was removed from the gel/blot sandwich and placed into a 1X PBS filled tray and agitated on a plate shaker for 5 minutes.

2.3.4. Antibody detection

The PBS was removed and 10mls of appropriate blocking buffer (table **2.1**) containing 1g of milk powder (1%), to block binding of non-specific protein, was placed on the nitrocellulose membrane and agitated for 1 hour. After sufficient exposure, the blocking buffer solution was replaced with the appropriate dilution of primary antibody plus blocking buffer solution (table **2.1**) and the membrane was incubated in primary antibody solution overnight at 4°C under gentle agitation. After overnight incubation, a 5 minute wash with PBS/Tween 20 (0.05%) (thrice) preceded removal of the primary antibody. The secondary antibody (HRP-conjugated) was diluted in blocking buffer (1:2000) and was added to the nitrocellulose membrane for one hour at room temperature. The membrane was washed and a 1:1 ratio of mixed Oxidising Reagent Plus and Enhanced Luminal Reagent Plus (Western Lightning-Plus ECL chemiluminescence detection kit) was applied evenly to the membrane for 2 minutes under agitation. The membrane was placed in a clear plastic cover and all air bubbles were smoothed out. The membrane was placed inside a UVP ChemiDoc-it imaging system and membranes were exposed to chemiluminescence. Exposure time was adjusted to get optimal band intensity across blots. Images were created using the VisionWorksLS image acquisition and analysis software package and saved as TIFF files. TIFF files were

opened in ImageJ 1.42 image software, and band densitometry was analysed by arbitrarily drawing a rectangular box around the selected protein bands of interest. The same rectangular box was used to measure all bands of interest and band intensities were quantified by conversion to profile plot histograms. By specifically selecting the peak of interest and closing off the peak with the line tool, background-subtracted density of each peak of interest is quantified. All densitometry data is normalised to the band intensity of the loading control used.

2.3.5. Loading control

Housekeeping proteins are useful loading controls for the western blot protocol. Following chemiluminescence, the nitrocellulose membrane was washed with 1X PBS and placed on a plastic tray and covered with stripping buffer (7.6g/l Tris HCL, 7ml 2-mecaptoethenol, 20g/l SDS, pH 6.7). The nitrocellulose membrane was incubated at 50°C in a shaking incubator to remove primary and secondary antibody interactions from the membrane. After repeated washing (six times) of the membrane in PBS/Tween 20 solution, the membrane was blocked in blocking buffer solution and re-probed with an appropriate dilution of housekeeping antibody (α-tubulin) plus blocking buffer solution (table **2.1**) for one hour. After incubation, the nitrocellulose membrane was washed and the chemiluminescence procedure was repeated.

To define the profiles a rectangular box was defined arbitrarily (except for testing the effects of the sample tool width; cf. below) with a width smaller than the narrowest band on the film and as long as the complete lane (cf. Fig. 2). Once defined, the same box was used to measure all lanes in both images.

Primary antibody/ MW	Manufacturer (cat. no.)	Buffer	Blocking buffer	Primary antibody/ buffer	Secondary antibody/ buffer	Loading control
Caspase-3 17-19 kDa	Sigma-Aldrich (C8487)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	GAPDH Sigma - Aldrich (G9295) 1:5000
COL2A1 190 kDa	Santa-Cruz (sc-7764)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
SOX9 65 kDa	Chemicon (AB5535)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
COMP 110 kDa	Kind gift from Dr J. Dudhia	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
HIF-1α 93 kDa	Abcam (ab51609)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:2000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
SOD1 23 kDa	Enzo (ADI-SOD-100)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
SOD2 25 KDa	Enzo (ADI-SOD-111)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
KEAP1 69 kDa	Santa-Cruz (sc-33569)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
NRF-2 68 kDa	BIOSS (BS-1074R	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	α-Tubulin Abcam (ab4074) 1:1000
α-tubulin	Abcam (ab4074)	PBS/T (0.05%)	5% milk powder/ PBS	5% Milk powder/ PBS/T 1:1000	Goat anti-rabbit HRP 1:2000	N/A

Table 2.1. Details of antibodies used in Western blotting protocols.

2.5. Measurement of Media pH

Media pH was measured after experimental incubations. An aliquot of spent media from each condition was taken and the extracellular pH measurements were recorded using a calibrated pH meter (Five Easy pH meter, Mettler Toledo, Leicester, UK).

2.6. Oxygen measurements

Measurements of O_2 tension were estimated using a modified version of the protocol outlined by Heimberg et al (2005). O_2 tensions from experimental media were measured using an O_2 electrode consisting of a platinum cathode and a silver anode embedded in an epoxy resin disc (Hansatech Instruments, Norfolk, UK) (figure **2.3**). An electrolyte saturated (KCl) piece of $2cm^2$ cigarette paper was placed over the dome shaped electrode smoothly, ensuring no creases were present, to create a uniform connection between cathode and anode. A $2cm^2$ piece O_2 permeable Teflon membrane was placed over the electrode, on top of the cigarette paper and was held in place using a sealing rubber ring. KCl was carefully pipetted into the reservoir surrounding the electrode, covering the anode. The measuring chamber was attached to the electrode and a small magnetic stirrer was placed inside the chamber. To analyse the O_2 concentration of a given solution, 1ml is pipetted into the chamber and the plunger is inserted down the chamber until it touches the surfaces of the solution (see figure **2.3**).

When a potentiating voltage is applied across the two electrodes the platinum becomes negative (cathode) compared to the silver which becomes positive (anode). Oxygen diffuses through the O_2 permeable Teflon membrane and becomes reduced at the cathode. Current flows due to the layer of electrolyte (KCl) covering the dome and that present in the reservoir (completing the circuit), ultimately oxidising the silver at the anode. The current generated is proportional to the O_2 reduced at the cathode which is then converted to a digital output on screen.

Prior to experimental media analysis, calibration of the measuring system was performed. N_2 flushed saline (0% O_2) was used to calibrate the system to zero. O_2

flushed saline (21% O_2) was used to calibrate the system for a maximum, fully saturated O_2 value. For experimental analysis, one ml of spent media from each condition was used. Currencies detected by the electrode were measured in triplicate and O_2 tensions were calculated using the equation:

$$\frac{\mathbf{A} - \mathbf{B}}{\mathbf{21}} = \mathbf{C}$$

$$\frac{\mathbf{D} - \mathbf{B}}{\mathbf{C}} = \mathbf{X}\ \%\ O_2$$

Where:

A = O_2 flushed saline

B = N_2 flushed saline

D = Value from unknown media sample

X = Actual O_2 tension value from unknown sample

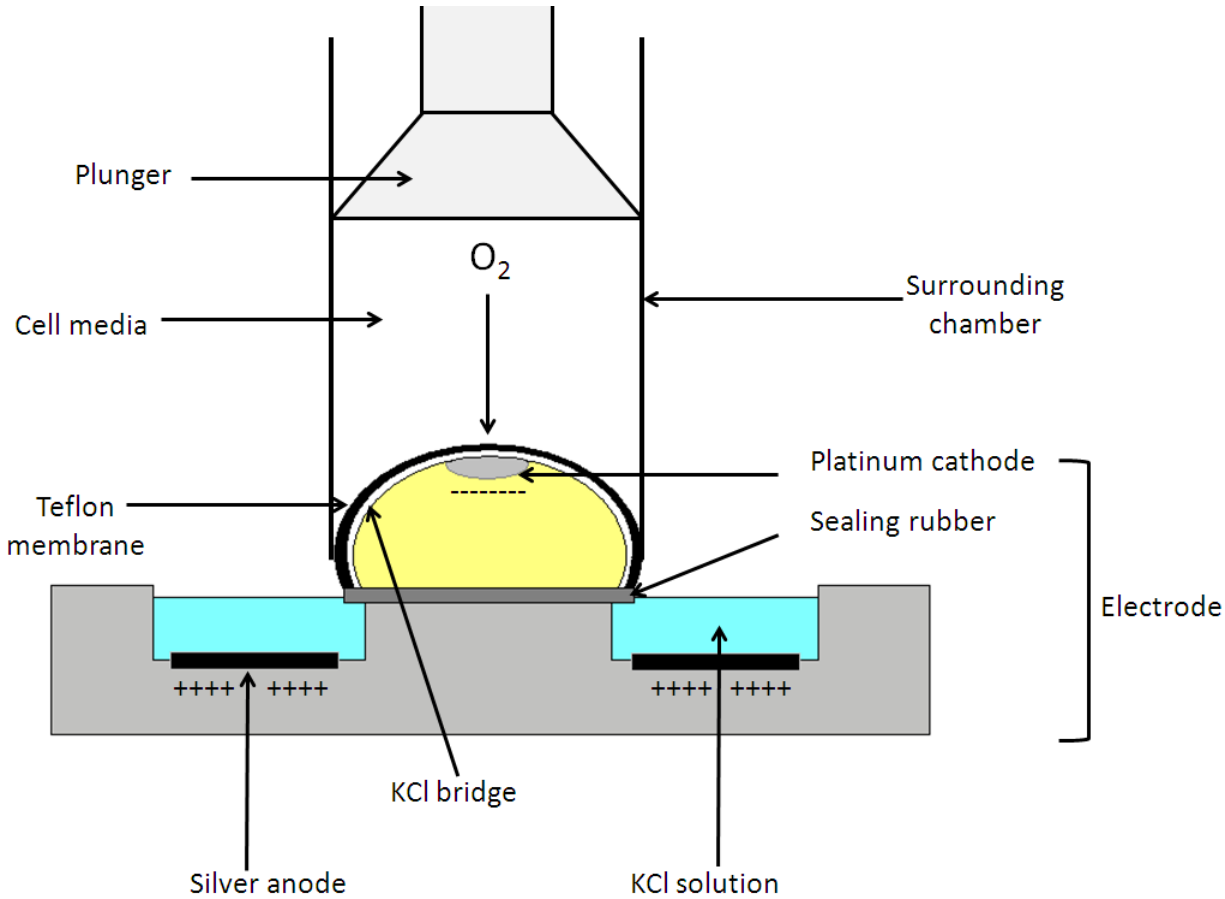


Figure 2.3. Hansatech Clarke type electrode setup for measurement of O_2 tensions in experimental media. The platinum cathode is located on the top of the electrode (dome) and is surrounded by a silver anode below it. A layer of electrolyte (KCl) connects the electrodes and is trapped under an O_2 permeable Teflon membrane. A solution of unknown O_2 concentration is entered into the chamber and the plunger is inserted down until it is in contact with the solution. If a current is given to the electrodes the cathode becomes negatively charged and the anode becomes positive. O_2 diffuses through the O_2 permeable membrane and is reduced at the cathode. Simultaneously, the anode is oxidised. The current passing through the electrode is converted to a numeric value which is proportional to the amount of O_2 in the sample. Using calibrated values from O_2 flushed saline (21% O_2) and N_2 flushed saline (0% O_2), unknown O_2 tensions were determined.

2.7. Measurement of glycosaminoglycan release

The dimethylmethylene blue (DMMB) assay was used to measure the concentration of sulfated GAG in spent media samples (Farndale et al, 1986). The DMMB assay is based on the shift in absorption observed when the DMMB dye associates with the negatively charged sulphates from released GAGs. This leads to binding of dye molecules and a metachromatic shift which can be measured spectrophotometrically (Mort & Roughley, 2007).

Initially a standard curve was produced using chondroitin sulphate C from Shark as a standard. Standards were serial dilutions ranging from 0-70µg/ml. In triplicate, 40µl of each standard and 40µl from each spent media sample were entered into a transparent flat bottomed 96-well plate. From a stock solution of DMMB dye (16mg 1-9 dimethyl methylene blue, 2g sodium formate, 2ml formic acid, made up to 1 litre in ddH_2O, pH 3.5), 250µl was pipetted into each media sample and standard. Absorbance was immediately read on a spectrophotometer at an absorbance of 570nm. The linear regression equation yielded from the standard curve allowed for determination of GAG concentrations from the unknown media samples (figure **2.4**).

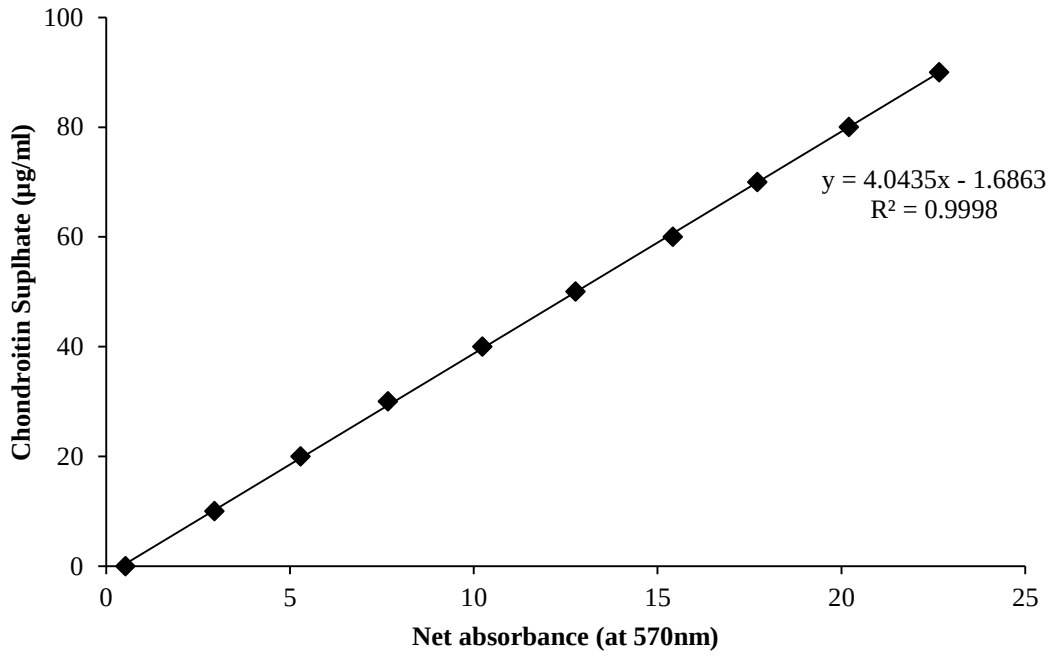


Figure 2.4. Typical standard curve for glycosaminoglycan quantification in spent media. Final concentrations of chondroitin sulphate (40 µl) ranging from 0-70 µg/ml were entered into wells of a 96 well plate in triplicate and 250µl of DMMB assay dye was added into each well. Absorbance was immediately read on a spectrophotometer at an absorbance of 570nm.

2.8. MMP-13 enzyme-linked immunosorbent assay (ELISA)

Total MMP-13 present in conditioned media was quantified using the MMP-13 Human ELISA Kit. A standard curve was produced using recombinant human MMP-13 as a standard. In triplicate, 100µl of each standard and media sample were entered into appropriate wells of a 96 well microplate coated with anti-human MMP-13. Wells were covered and incubated for 2.5 hours at room temperature. Standards and media samples were discarded and all wells were washed with wash buffer before 100 µl of biotinylated antibody was added to each well. Microplates were incubated for one hour under gentle agitation at room temperature. Removal and washing of all wells preceded the addition of 100µl prepared Streptavidin solution to each well. The microplate was incubated for 45 minutes under gentle agitation at room temperature. Following removal of Streptavidin, all wells were incubated for 30 minutes in 100µl TMB One-step Substrate Reagent. TMB One-step Substrate Reagent was completely removed, 50µl of Stop Solution was added to each well and microplates were immediately read at a wavelength of 450nm. The linear regression equation yielded from the standard curve allowed for determination of released active-MMP-13 from spent media samples.

2.9. Intracellular ATP (ATP_i) measurement

Intracellular ATP levels were quantitatively assessed using the EnzyLight™ ATP Assay Kit as specified by the manufacturer's instructions. A standard curve was produced using ATP as a standard. In triplicate, 10µl of each standard and 10µl of cell suspension were entered into a white opaque 96 well plate and 90µl of Reconstitution Reagent (containing assay buffer, substrate and ATP enzyme (95:1:1) was added into each well. Luminescence was immediately read on a Bio-Tek FLX800 luminescent plate reader. The linear regression equation yielded from the standard curve (figure **2.5**) allowed for determination of intracellular ATP concentrations from chondrocyte cell suspensions.

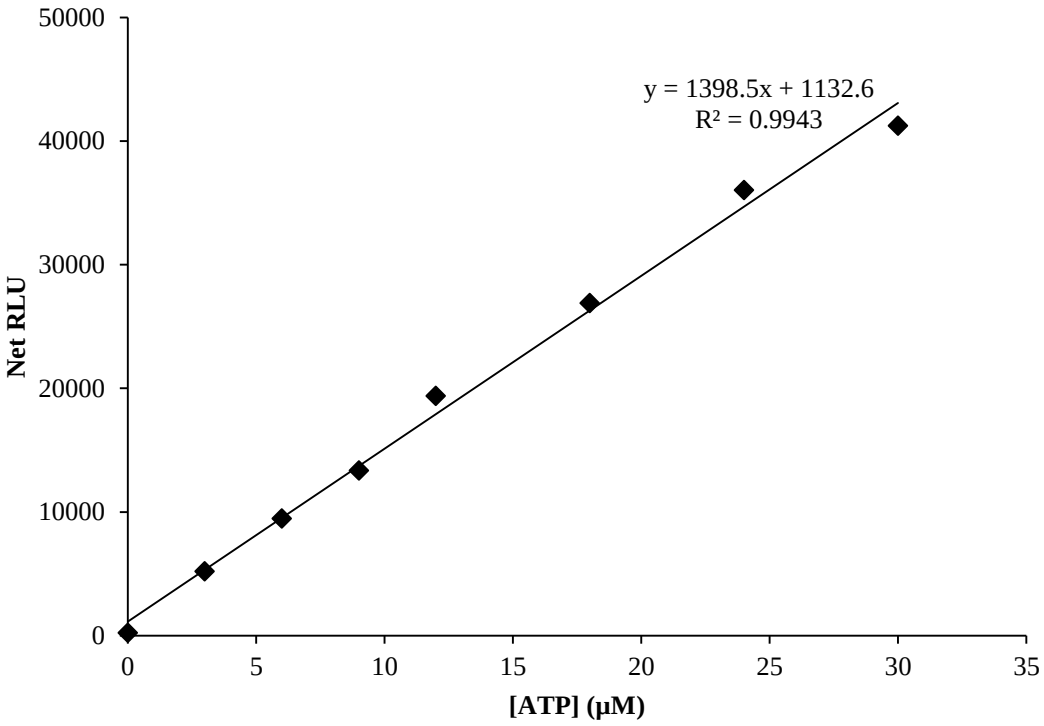


Figure 2.5. Typical standard curve for intracellular ATP quantification in chondrocytes. Final concentrations of ATP (10 µl) ranging from 0-30 µg/ml were entered into wells of a 96 well plate in triplicate and 90µl of reconstitution reagent was added into each well. Luminescence was immediately read on a luminescence plate reader.

2.10. Measurement of Nitric Oxide production

Nitric oxide production was analysed by measuring the stable metabolic end products of nitric oxide release (nitrite and nitrate) in spent culture media. Nitrite and nitrate was quantified using the Griess reaction with the Nitrite/Nitrate Assay Colorimetric Kit according to manufacturer's instructions. The Griess assay mechanism is based on the principle that nitric oxide processing forms nitrite production which reacts with sulphanilamide to form a diazonium salt that can be coupled to naphthylethylenediamine to create a stable water soluble dye that can be measured at 570 nm. The amount of dye formed is proportional to the amount of nitrite in the unknown sample (Hetrick & Schoenfisch, 2009).

Initially, two calibration curves were produced. Firstly, a nitrite calibration curve was produced using final concentrations of sodium nitrite ($NaNO_2$) ranging from 25-100 µM (figure **2.6 A**). Secondly, a nitrate + nitrite calibration curve was produced using final concentrations of Sodium Nitrate ($NaNO_3$) ranging from 25-100 µM (figure **2.6 B**). Using the linear regression equations from the standard curves, the determinations of nitrite and nitrate concentrations from the unknown media samples were calculated.

2.10.1. Determination of nitrite from media samples

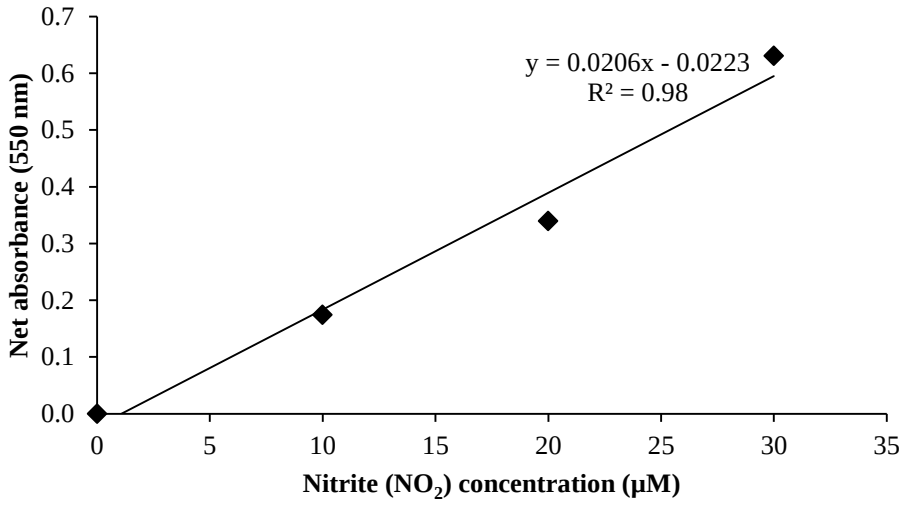
For determination of nitrite values, 80 µl of each media sample was added in triplicate to wells of a 96-well plate and the supplied buffer solution was added to each well to a volume of 100 µl. Griess reagent A (*N*-(1-naphthalenediamine) (50 µl) was added to each media sample and the plate was incubated for 5 minutes at room temperature. Fifty microlitres of Griess reagent B (Sulfanilic acid) was pipetted into each well and an incubation period of 10 minutes at room temperature preceded an absorbance measurement at a wavelength of 550nm using a photometric microplate absorbance reader. The blank value determined whilst producing the standard curve for nitrite was subtracted from all absorbance values and nitrite concentrations for each unknown media sample was calculated (figure **2.5 A**).

2.10.2. Determination of nitrate from media samples

To determine nitrate values, nitrate must first be reduced to nitrite utilising nitrate reductase. Eighty microlitres of each media sample was added in triplicate to wells of a 96-well plate. Ten microlitres of both nitrate reductase and enzyme co-factor solution was added to each well and the plate was incubated at 25˚C for 2 hours. Griess reagent A (50 µl) was added to each media sample and the plate was incubated for 5 minutes at room temperature. Fifty microlitres of Griess reagent B was pipetted into each well and an incubation period of 10 minutes at room temperature preceded an absorbance measurement at a wavelength of 550nm. The blank value determined whilst producing the standard curve for nitrite + nitrate was subtracted from all absorbance values and nitrite + nitrate concentrations for each unknown media sample was quantified (figure **2.5 B**). Determination of nitrate concentration was then calculated using the following equation:

$$[\text{Nitrate}] = [\text{Nitrate} + \text{Nitrite}] - [\text{Nitrite}]$$

A



B

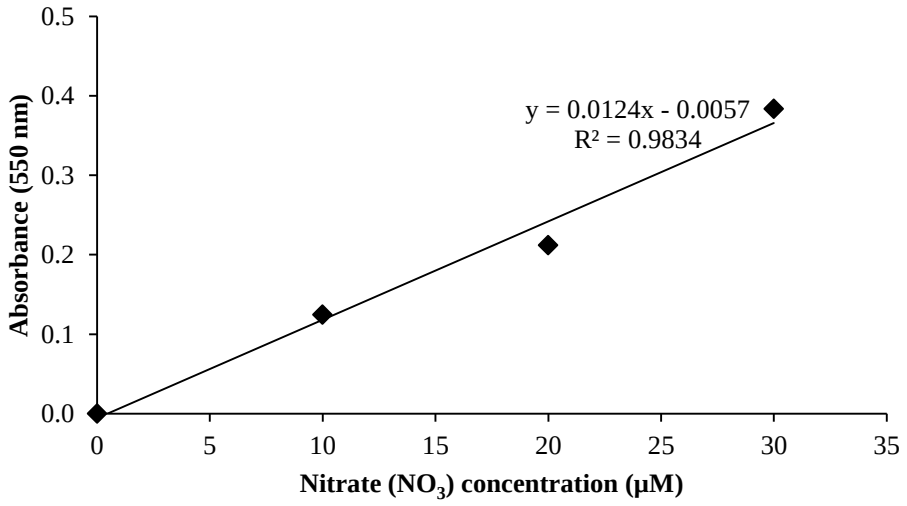


Figure 2.6. Calibration curves for nitrite (A) and nitrate (B) quantification. For the nitrite calibration curve (**A**): Final concentrations of Sodium Nitrite ($NaNO_2$) ranging from 25-100 µM were entered into wells of a 96 well plate and made to 100µl with supplied buffer solution. Griess reagent A (50µl) was added to each media sample and the plate was incubated for 5 minutes at room temperature. 50µl of Griess reagent B was pipetted into each well and an incubation period of 10 minutes at room temperature preceded an absorbance measurement at a wavelength of 550 nm. For the nitrate calibration curve (**B**): Final concentrations of Sodium Nitrate ($NaNO_3$) ranging from 25-100 µM were entered into wells of a 96 well plate. 10µl of both nitrate reductase and enzyme co-factor solution were added to each well and the plate was incubated at room temperature for 2 hours. Griess reagent A (50 µl) was added to each media sample and the plate was incubated for 5 minutes at room temperature. 50µl of Griess reagent B was pipetted into each well and an incubation period of 10 minutes at room temperature preceded an absorbance measurement at a wavelength of 550 nm.

2.11. Measurement of lactate dehydrogenase (LDH) as a marker of cell viability

Cell viability is commonly evaluated by measuring plasma-membrane damage. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. During plasma membrane damage or cellular apoptosis, LDH is rapidly released into the culture medium *in vitro*. LDH enzyme activity in culture supernatant increases as the number of non-viable cells (or cells with damaged plasma membranes) increases, thus measurement of media LDH is commonly used to give an estimation of cell viability (Haslam et al, 2000).

Cell viability was quantified using the Cytotoxicity Detection Kit ^{Plus} as per the manufacturer's instructions. It is based on the principle that an increase in dead or plasma membrane damaged cells results in increased lactate dehydrogenase (LDH) release into cell culture media. Briefly, 100 µl of spent media from each condition was entered, in triplicate, into wells of a clear, flat bottomed, 96-well plate. 100 µl microlitres of reaction mixture (consisting of 1 ml of catalyst-diaphorase/NAD^+ mixture and 45 ml of dye solution-INT and sodium lactate) was added to each well and was protected from light and incubated for 30 minutes at room temperature. After incubation, dual absorbance measurements were immediately measured at wavelengths of 490nm and 600nm (reference wavelength) on a microplate reader. Values at 600nm were subtracted from all experimental well values and data was presented as percentage compared to control.

2.12. Quantification of reactive oxygen species (ROS) generation

Intracellular reactive oxygen species were measured using the Dichlorofluorescin (DCF) method (Chandel and Schumacker, 2000). The lipophilic dichlorofluorescin diacetate (DCFH-DA) freely diffuses into cells and is deacetylated to 2 ′ ,7 ′ -dichlorodifluorescein (DCFH), which becomes trapped within cells. Intracellular DCFH, a non-fluorescent fluorescein analogue, is oxidized by ROS to produce 2′ 7 ′ -dichlorofluorescein (DCF) which is highly fluorescent. DCFH-DA (100 mg) was diluted in anhydrous DMSO to give a 100mM stock concentration. Working concentrations (1:1000 dilutions) of 100 µM were used for ROS quantification.

Chondrocytes were seeded at $1x10^6$ cells/ml in pre-equilibrated isotonic saline and 100 µM DCFH-DA was added to cell suspensions and vortex mixed gently. A blank condition consisting of pH 7.2 isotonic saline (pre equilibrated to 5% O_2) plus cells in the absence of DCFH-DA was created to give an autofluorescence value which was subtracted from all experimental values. Cell suspensions containing DCFH-DA were incubated at the relevant O_2 concentration at 37°C for 40 minutes, after which cells were washed twice with 1X PBS (pre equilibrated to the relevant O_2 tension). Cells were then centrifuged at 394 rcf for 4 minutes and resuspended in 300µl pre equilibrated isotonic saline and 100µl of cell suspensions were entered into wells of a black clear bottomed 96-well plate in triplicate. DCF fluorescence was measured (excitation at 490nm/emission 535nm) using a microplate fluorescence/luminescence plate reader (FLX800, BioTek Instruments, Vermont, USA).

2.13. Measurement of the mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential was determined by the fluorescent probe 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Salvioli et al, 1997). If mitochondria are polarised, the lipophilic dye enters the mitochondrial matrix of cells where it accumulates forming fluorescent red aggregates. During mitochondrial depolarisation, JC-1 cannot accumulate and remains in its monomeric form in the cytoplasm and fluoresces green.

Chondrocytes were seeded at $1x10^5$ in pre-equilibrated isotonic saline and 50 µg/ml JC-1 was added to cell suspensions and vortex mixed gently. Cell suspensions were incubated in the relevant oxygen tension at 37°C for 40 minutes (humidified atmosphere). After incubation, cell solutions were centrifuged at 394 rcf for 4 minutes and the cell pellets were washed in pre equilibrated sterile isotonic saline. The cell solution was centrifuged at 394 rcf for 4 minutes and resuspended in 50µl VECTASHIELD™. 10µl of cell suspensions were pipetted onto individual coverslides and mounted with coverslips. Slides were viewed at a 10X magnification using a Nikon ECLIPSE TS-100 inverted microscope and fluorescence was seen using the ECLIPSE TS100/TS-100-F Epi-fluorescence attachment and Nikon digital

Sight photo port (Surrey, UK). Specific filters for rhodamine and flourescein were used to view red and green fluorescence respectively.

The red aggregates (polarised) emit at 585/90 nm. In its monomeric form JC-1 will appear green with an emission centred at 514/530 nm (indicating depolarisation). Three representative images per wavelength per condition were taken (n=3 donors, n=3 technical replicates). Acquired images were analysed and processed using ImageJ 1.42. The red:green fluorescence pixel intensity ratio was used to determine the polarisation of chondrocyte mitochondria exposed to each condition. A reduction in the ratio indicates a reduction in the mitochondrial membrane potential.

2.14. Quantification of the GSH:GSSG ratio

GSH and GSSG were individually quantified (in triplicate, from the same sample) using the GSH:GSSG-Glo™ Assay. This produces stable luminescent signals which correlate with individual measurements of the (reduced glutathione) GSH or (oxidised glutathione) GSSG content of a sample. For GSH measurement, total glutathione (both GSH and GSSG) is calculated by reducing total GSSG to GSH. Total GSH is then converted to luciferin via the GSH probe Luciferin N-T, catalysed by glutathione-S-transferase. Formation of luciferin is coupled to a firefly luciferase reaction, emitting light that is dependent on luciferin formation. Luciferin formation is in turn dependent on the amount of GSH present. Thus, the luminescent signal is proportional to the amount of GSH. For determination of GSSG in unknown samples, GSH content is initially inhibited and GSSG is reduced to form GSH. The GSH representing total GSSG is then subject to the luminescence reaction above to give a measure of GSSG.

The protocol followed manufacturer's instructions. Table **2.2** shows reagents prepared prior to analysis. All reagents were used within 30 minutes of preparation. Chondrocytes were seeded at $1x10^5$ in pre-equilibrated isotonic saline (see table **2.1**) and in triplicate, 20 µl of cell solution from each condition was entered into a luminometer compatible opaque white flat bottomed 96-well plate. Analysis of total glutathione and oxidised glutathione were assayed separately (allowing for

determination of the GSH:GSSG ratio). Twenty five microlitres per well of Total Glutathione Lysis Reagent or Oxidised Glutathione Lysis Reagent were added to the appropriate wells. The plate was shaken for 5 minutes on a plate shaker and then 50 µl/well of Luciferin Generation Reagent was added to all wells. The plate was shaken and incubated for 30 minutes at room temperature. Finally, 100 µl/well of Luciferin Detection Reagent was added to each well and the luminescence was immediately read using a microplate luminescence reader (FLX800, BioTek Instruments, Vermont, USA). The average values from a no-cell control (performed in triplicate) were subtracted from the average of all other wells and the GSH:GSSG ratio was determined using the following equation:

(Net treated total glutathione RLU – Net treated GSSG RLU) / [Net treated GSSG RLU/2]

Total Glutathione Lysis Reagent Concentrated)	
Component	**Volume per reaction (96-well plate)**
Luciferin-NT	1.0 µl
Passive Lysis Buffer, 5X	10.0 µl
Water	14.0 µl
Final volume per reaction	**25.0 µl**

Oxidised Glutathione Lysis Reagent (concentrated)	
Component	**Volume per reaction (96-well plate)**
Luciferin-NT	1.0 µl
NEM, 25mM	0.5 µl
Passive Lysis Buffer, 5X	10.0 µl
Water	13.5 µl
Final Volume per reaction	**25.0 µl**

Luciferin Generation reagent	
Component	**Volume per reaction (96-well plate)**
100mM DTT	1.25 µl
Glutathione-S-Transferase	3.0 µl
Glutathione Reaction Buffer	45.75 µl
Final Volume per reaction	**50 µl**

Luciferin Detection Reagent
Reconstitution Buffer with Esterase (supplied) transferred to Luciferin Detection Reagent (supplied) and mixed by inversion until the substrate is thoroughly dissolved.
Final Volume per reaction (96-well plate):100 µl

Table 2.2. Reagent preparation for the GSH:GSSG-Glo™ Assay. Total glutathione, oxidised glutathione (GSSG only) and luciferin generation and detection reagents were adjusted for the total number of reactions that were performed.

2.15. Measurement of Intracellular pH

2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) is the most extensively used dye for measurement of intracellular pH. The dye is facilitated into cells via an acetoxymethyl (AM) ester group (cell permeant BCECF-AM) which aids uptake by masking the carboxyl group on the dye (and hence its negative charge). The addition of AM esters to fluorescent probes results in an uncharged molecule capable of traversing cell membranes. BCECF-AM is non-fluorescent. Once inside, the acetoxymethyl group is cleaved by intracellular esterases to produce BCECF, a fluorescent compound. BCECF has 4-5 negative charges at physiological pH and is therefore retained within the cell. The excitation profile of BCECF is pH dependent. Emission is measured at 535nm following dual excitation at both 490 and 435 nm giving ratiometric values which reduce error margins associated with single wavelength measurements. Emission following excitation at 435nm is insensitive to pH (isosbestic point) whereas emission following excitation at 490nm is sensitive to pH. Therefore, an increase in the ratio measured at 535 nm following dual excitation indicates increased alkalinity (an increase in intracellular pH). Intracellular pH calibrations were carried out in order to determine intracellular pH values of chondrocytes.

2.15.1 BCECF calibration

The high K^+-nigericin method was used to measure intracellular pH for calibrations (Thomas et al, 1979). Nigericin is an ionophore and is used to clamp external and internal H^+ and K^+ concentrations. The following calculation was used where K^+_i is intracellular potassium, K^+_o is external potassium, H^+_i is intracellular H^+ and H^+_o is extracellular H^+.

$$\frac{[K^+]_o}{[H^+]_o} = \frac{[K^+]_i}{[H^+]_i}$$

Using this equation, intracellular pH was calculated by measuring external pH using a known concentration of K^+_o (145mM = $[K^+]_i$).

Four aliquots of chondrocytes were seeded at $1x10^6$ in isotonic saline with 10µM BCECF-AM and incubated for 30 minutes at 37°C (humidified atmosphere). After incubation, cell solutions were centrifuged at 394 rcf for 4 minutes and the supernatant removed. Each aliquot was then resuspended in high K^+-nigericin media (see table **2.3**) for 10 minutes at 37°C (humidified atmosphere). Extracellular pH measurements from each aliquot were taken using a calibrated pH meter. Each cell suspension was then loaded into a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan) and fluorometric analysis was performed. Dual measurements were taken in triplicate (Ex: 490/435; Em: 535 nm). Cell suspensions were then centrifuged and each aliquot was resuspended in one of four pre titrated pH saline solutions; pH 6.0, pH 6.5, pH 7.0, or pH 7.5 and allowed to equilibrate for 10 minutes. New ratiometric measurements were then taken in triplicate (Ex: 490/435; Em: 535 nm) and average values were calculated and a calibration curve was created. The linear regression equation obtained using this data was used to calculate intracellular pH in unknown samples (figure **2.7**).

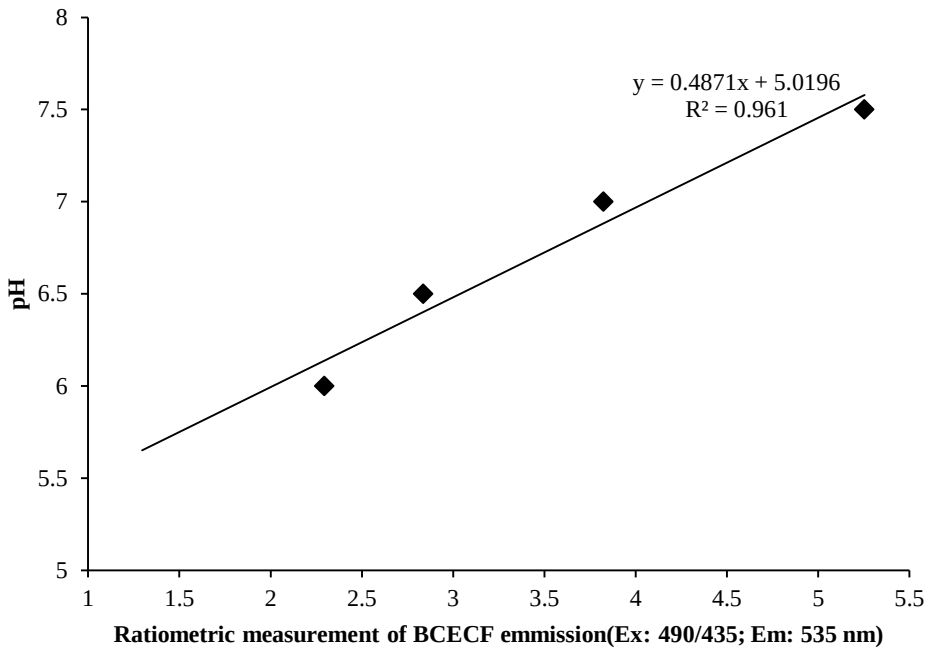


Figure 2.7. Calibration of pH_i using the high K^+-nigericin method. Chondrocytes ($1x10^6$) were incubated with 10µM BCECF-AM for 30 minutes at 37°C. Cell solutions were centrifuged and cells were resuspended in high K^+-nigericin saline (see table **2.3**) for 10 minutes at 37°C. Extracellular pH was recorded in high K^+-nigericin suspensions. Cell suspensions were then loaded into a fluorescence spectrophotometer and fluorometric analysis was performed. Dual measurements were taken in triplicate (Ex: 490/435; Em: 535 nm). Cell suspensions were then centrifuged and each aliquot was resuspended in varying pH saline solutions (pH 6.0, pH 6.5, pH 7.0, or pH 7.5) and allowed to equilibrate for 10 minutes. Ratiometric measurements were repeated and average values were calculated. The linear regression equation obtained using this data was used to calculate pH_i in unknown samples.

2.15.2. Steady state intracellular pH measurement

Chondrocytes were seeded at $1x10^6$ in pre-equilibrated isotonic saline and incubated with 10µM BCECF-AM for 30 minutes at the relevant oxygen concentration at 37°C (humidified atmosphere). Cells were then centrifuged at 394 rcf for 4 minutes and washed twice in isotonic saline. BCECF fluorescence was spectrophotometrically measured in triplicate (excitation at 490nm, emission at 535nm) using an FLX800 microplate fluorescence reader. The average value from each condition was calculated and a value for intracellular pH was determined using the formula gained from the intracellular pH calibration curve.

2.16. Statistical analysis

All data was analysed using IBM SPSS Statistics 20. Normal distribution for each data set was assessed using the Kolmogorov-Smirnov test for normality. All data sets were normally distributed. Statistical analysis was performed on all data sets and significant differences were determined by One-way ANOVA with a Dunnett's and Tukey HSD *post-hoc* correction. Results are presented as mean values ± standard error of the mean (SEM). Exact P-values are presented for all data sets as appropriate. A significance level of $P<0.05$ was used. Unless otherwise stated, results are presented as % compared to control (5% O_2, pH7.2, time = 0).

Isotonic Saline (Bicarbonate free) isotonic saline		
Compound	**Concentration (mM)**	**Amount required (g/l)**
NaCl	145	8.47
KCL	5	0.37
$MgSO_4.6H_2O$	1	0.2
$CaCl_2$	2	2 ml (of 1M solution)
MOPS	10	2.09
D-glucose	10	1.80

High K^+ (Bicarbonate free) isotonic saline		
Compound	**Concentration (mM)**	**Amount required (g/l)**
KCl	145	10.82
$MgSO_4.6H_2O$	1	0.2
MOPS	10	2.09
D-glucose	10	1.80
$CaCl_2$	2	2 ml (of 1M solution)

Table 2.3. Composition of sterile solutions used in experiments. All saline's were titrated to pH 7.4 using NaOH and HCL. For high K^+ saline, isotonic saline was used with added K^+ (145mM) and 2µg/ml nigericin in the absence of NaCl.

Chapter 3

Development of a 3-D alginate bead cell culture model to investigate the cellular responses to variations in O_2 tension and pH in human osteoarthritic articular chondrocytes (HOAC).

Aspects of this chapter have been published (see **appendix ii** for full article):

Collins, JA., Moots, RJ., Winstanley, R., Clegg, PD., Milner, PI., (2013). Oxygen and pH-sensitivity of human osteoarthritic chondrocytes in 3-D alginate bead culture system. *Osteoarthritis and Cartilage* [ahead of print] DOI: 10.1016/j.joca.2013.06.028

3.1. Introduction

Mature cartilage is avascular, relatively hypoxic and acidic and thus provides an unusual and challenging environment to the resident cell, the chondrocyte (Wilkins et al, 2000). The avascular nature of cartilage results in chondrocytes being exposed to relatively low concentrations of oxygen compared to other tissue types, with diffusion gradients existing through cartilage from around 7.5% at the surface, to around 2% in the deeper layers (Zhou et al, 2004). In addition to this hypoxic environment, articular chondrocytes are embedded within an extracellular matrix rich in collagen type II and proteoglycans. The fixed negatively charged proteoglycans are responsible for the compressive stiffness of articular cartilage, by attracting free mobile cations (e.g. Na^+, H^+) and osmotically obliged water, resulting in a hydrated matrix of increased osmolarity and reduced pH (Hardingham & Forsang, 1992). As well as the acidic extracellular environment, production of lactate from predominately anaerobic glycolytic metabolism leads to increased intracellular acid loads experienced by chondrocytes.

In joint diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA), oxygen levels are reduced via increased cellular oxygen consumption (such as by synoviocytes) and reduced delivery of oxygen to synovial fluid due to joint capsule fibrosis and subchondral bone sclerosis (Biniecka et al, 2010; Levick, 1990). Alterations in the physical environment and release of inflammatory mediators (such as pro-inflammatory cytokines, nitric oxide, prostaglandins) by articular cells, under disease conditions lead to acidosis (Mapp et al, 1995; Konttinen et al, 2002). Indeed matrix pH is decreased in degenerative joint diseases and correlates with inflammation (Farr et al, 1985; Kofoed, 1986). A lowered extracellular pH can also increase the activity of proteases (such as MMP-13) which can exacerbate joint destruction (Christensen et al, 2005). It has been shown that ECM synthesis is pH sensitive and even minor fluctuations in extracellular pH from within the physiological range can significantly modulate ECM components (Das et al, 2010; Wilkins and Hall, 1995; Wu et al, 2007).

Since matrix synthesis and activity of many degradatory enzymes are oxygen and pH-sensitive (Coyle et al, 2005; Schneider et al, 2005), changes in the physicochemical environment of the chondrocyte are likely to have important effects on chondrocyte function. Attenuation of the mechanisms involved in intracellular pH regulation has been previously demonstrated under short-term hypoxic conditions and highlights the modulatory effect of changes to oxygen tension on chondrocyte homeostasis (Gibson et al, 2008; Milner et al, 2006). The interaction of oxygen and extracellular pH on chondrocyte function across (patho) physiological ranges likely to occur in healthy and diseased cartilage has not been fully characterised.

The notion of hypoxic intracellular signalling is a critical parameter influencing chondrocyte function (Henrotin et al, 2005). Many *in vitro* studies fail to culture chondrocytes in O_2 tensions representative of the joint and may not consider the effects of extracellular pH on chondrocyte function. Additionally, choice of cell culture technique and appropriate media buffering systems must be taken into account when investigating chondrocyte responses *in vitro*.

In an effort to appropriately analyse the effects of O_2 tension and pH on chondrocyte function *in vitro*, it is necessary to develop a model taking these factors into account. Accordingly, the work presented in the following chapter describes the development of a three dimensional *in vitro* chondrocyte model revolving around varying O_2 tensions and pH. Such a model aims to mimic the range of physiological conditions present in the diseased and non-diseased joint. This should provide insights into intracellular dysfunction in joint disease, enhancing our understanding of affected pathways in cells exposed to sustained physiological stress.

3.2. Aims

The aims of this part of the project were:

(1) To identify the effect of alterations in oxygen tension and extracellular pH on HOAC cell function cultured in a three dimensional alginate bead model over a 96 hour time course.

(2) To assess the effect of the pro-inflammatory cytokine IL-1β, a well-known catabolic cytokine in the perpetuation of OA, on HOAC function exposed to physical parameters such as oxygen and pH over a 96 hour time course.

3.3. Study Design

- **Cell culture and experimental culture conditions**

Human osteoarthritic articular cartilage was obtained from knee joints removed by total-knee arthroplasty. Tissue was obtained from a total of 3 donors (3 male aged 68-77, mean age; 71 ± 4.93 years). HOAC was isolated by enzymatic digestion and cells were maintained and expanded in continuous monolayer culture in a 5% O_2 humidified environment with HEPES buffered media pre-titrated to pH 7.2. To prevent dedifferentiation to a fibroblastic morphology with subsequent loss of chondrocytic phenotype, monolayer cultured cells were trypsinised and cultured in three-dimensional alginate bead culture by passage 3 (Tew et al, 2008). Alginate beads were incubated and maintained in a 5% O_2 tension for 14 days. On day 14, a sample of alginate beads (n=3) were depolymerised via sodium citrate extraction. Released cells were either immediately analysed or lysed in SDS lysis buffer prior to western blotting analysis (t=0). Remaining alginate beads were seeded into 6 well plates and were incubated in DMEM titrated to pH 7.2 (normal media) or pH 6.2 (acidic media) in the absence or presence of IL-1β (10 ng/ml) for 24, 48 or 96 hours in <1%, 2%, 5% or 21% O_2 conditions (figure **3.1**). Following 24, 48 or 96 hours experimental incubation, O_2 and extracellular pH measurements of the media were taken and the remaining media sample frozen for analysis of GAG, nitrite and nitrate release. Alginate beads were released from their alginate matrix by depolymerisation and immediately analysed.

- **Identification of a chondrocyte phenotype by Western blotting**

The chondrocyte phenotype was characterised by analysing protein expression of COL2A1 and COMP (see materials and methods for antibody details (table **2.1**)). The house-keeping gene α-tubulin was used as a loading control.

- **Glycosaminoglycan (GAG) release**

The dimethylmethylene blue (DMMB) dye binding assay was used to assess the concentration of sulfated GAG in spent media samples. Absorbance of unknown

spent media samples was spectrophotometrically measured at 570nm and GAG concentrations from each condition were determined using GAG calibration curves using chondroitin sulphate as a standard.

- **Nitric Oxide production**

Nitric Oxide (NO) production was colorimetrically determined by measuring the stable metabolic end products of nitric oxide release (nitrite and nitrate) in the spent culture media. Nitrite and nitrate was quantified using the Griess reaction with the Nitrate/nitrite Assay Colorimetric Kit according to manufacturer's instructions. The concentration of both nitrite and nitrate were estimated from standard curves which were produced using sodium nitrite and sodium nitrate as standards.

- **Cell viability**

Cell viability was evaluated using the trypan blue exclusion method. Chondrocytes were washed, resuspended in sterile PBS with Trypan Blue dye solution. Percentage cell viability was determined manually, by counting the number of cells in which the dye had traversed the membrane, staining the cell blue (indicative of membrane damage and cell death) and the number of cells that excluded the dye (intact cell membranes and therefore viable).

- **Intracellular ATP (ATP_i) measurement**

Intracellular ATP levels were quantitatively assessed using the EnzyLight™ ATP Assay Kit as specified by the manufacturer's instructions. Luminescence was immediately read and the linear regression equation yielded from the standard curve allowed for determination of intracellular ATP concentrations from chondrocyte cell suspensions.

- **Active MMP-13 enzyme-linked immunosorbent assay (ELISA)**

Active MMP-13 present in conditioned media was quantified using the MMP-13 Human ELISA Kit according to manufacturer's instructions. A standard curve was

produced using recombinant human MMP-13 which was used to determine the levels of MMP-13 in cell culture supernatants.

- **Statistical analysis**

Significant differences were determined by One-way ANOVA with a Dunnett's and Tukey HSD *post-hoc* correction. Results are presented as mean values ± SEM. Exact P-values are presented. A minimal significance level of $P<0.05$ was used. Unless otherwise stated, results are presented as % compared to control (5% O_2, pH7.2, time=0).

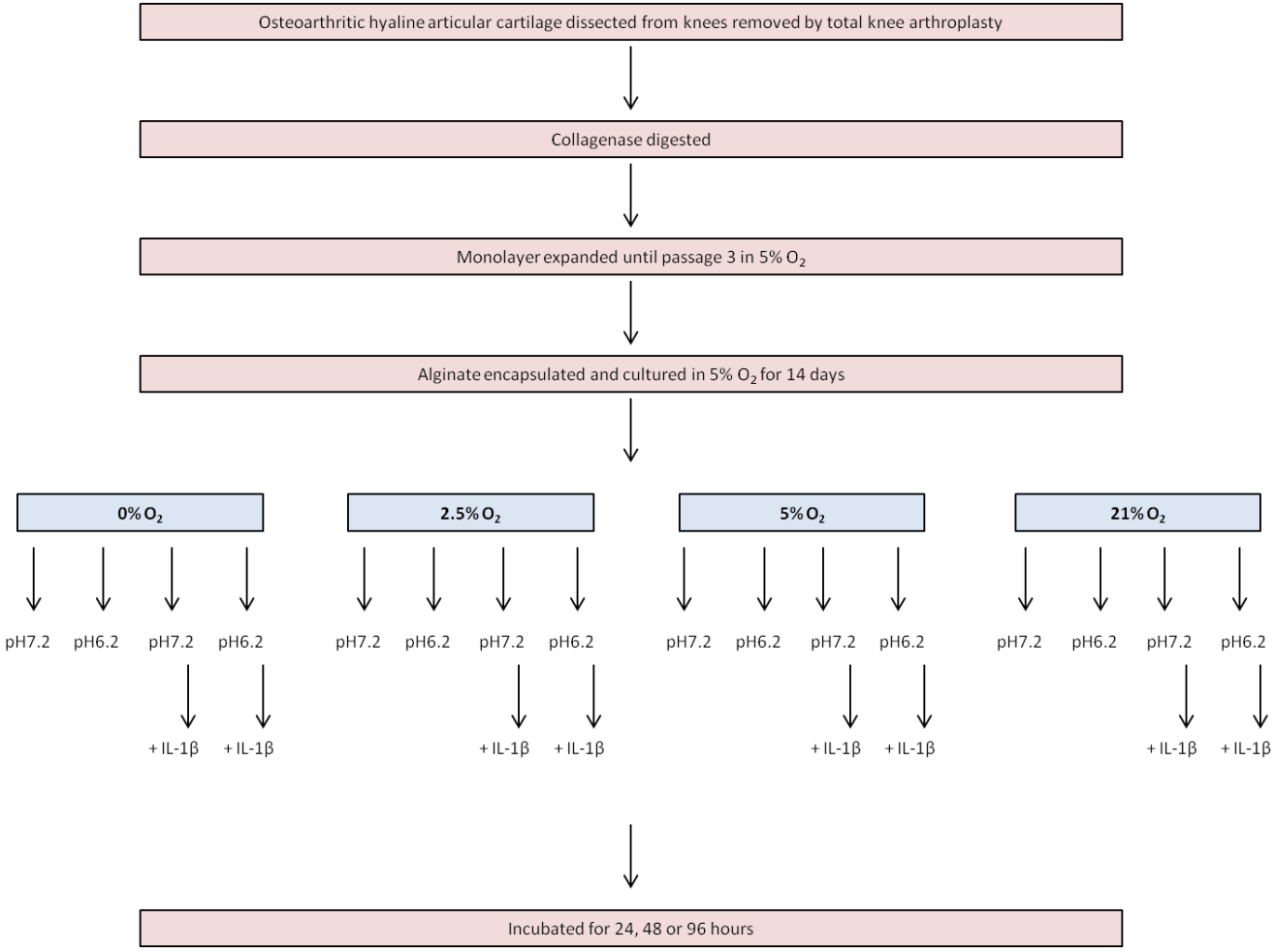


Figure 3.1. Schematic detailing treatments for HOAC cultured in alginate beads. Alginate beads were cultured in normal (pH 7.2) or acidic (pH 6.2) media in the presence or absence of 10ng/ml IL-1β for 24, 48 or 96 hours in one of four O_2 tensions: 21%, 5%, 2.5% or <1%.

3.4. Results

Despite the *in vivo* hypoxic nature of human articular cartilage and the gradients of O_2 that exist therein, the majority of chondrocyte analysis *in vitro* is undertaken at possibly inappropriate ambient O_2 levels of 21%. This represents hyperoxic conditions to chondrocytes. Similarly, *in vivo* cartilage is acidic compared to many other tissues. The current investigations set out to determine if varying O_2 tensions and extracellular pH altered osteoarthritic chondrocyte responses *in vitro*.

3.4.1. Chondrocyte phenotype and media conditions

In order to ensure the pH of cell culture media remained to pre-titrated values across the time course investigated, a preliminary investigation into the efficacy of three buffers to maintain pH was conducted. HEPES and PIPES buffers were chosen for titration of cell culture media to pH 7.2 and pH 6.2 owing to their linear buffering capacity falling between pH 6.8-8.2 and pH 6.0-7.0 respectively. The efficacy of these buffers to maintain pH was compared to that of the commonly used bicarbonate (HCO_3^-) buffered Dulbecco's Modified Eagles Media (DMEM). Table **3.1** shows the ability of HEPES and PIPES buffered media to maintain media pH to pre-titrated values. HCO_3^- buffered media led to media alkalinisation at all O_2 tensions studied. As a result, HEPES (for media titrated to pH 7.2) and PIPES (for media titrated to pH 6.2) buffered DMEM were used for all experiments presented in this project.

2.5% O_2				
	HCO_3^- buffer (pH 7.2)	**HEPES buffer (pH7.2)**	**HCO_3^- buffer (pH 6.2)**	**PIPES buffer (pH 6.2)**
0	7.22 ± 0.28	7.27 ± 0.01	6.22 ± 0.02	6.23 ± 0.03
24	7.99 ± 0.09 †	7.29 ± 0.02	6.97 ± 0.01 †	6.24 ± 0.02
48	8.10 ± 0.07 †	7.33 ± 0.04	7.38 ± 0.02 †	6.30 ± 0.01
96	8.18 ± 0.04 †	7.30 ± 0.02	7.58 ± 0.10 †	6.33 ±0.02

5% O_2				
	HCO_3 buffer (pH 7.2)	**HEPES buffer (pH7.2)**	**HCO_3^- buffer (pH 6.2)**	**PIPES buffer (pH 6.2)**
Time (hrs)	**pH**	**pH**	**pH**	**pH**
0	7.24 ± 0.09	7.20 ± 0.01	6.23 ± 0.04	6.24 ± 0.03
24	7.88 ± 0.12 †	7.26 ± 0.03	6.57 ± 0.11 †	6.21 ± 0.02
48	7.94 ± 0.06 †	7.28 ± 0.04	7.49 ± 0.05 †	6.28 ± 0.01
96	7.99 ± 0.21 †	7.27 ± 0.03	7.66 ± 0.09 †	6.31 ± 0.01

21% O_2				
	HCO_3^- buffer (pH 7.2)	**HEPES buffer (pH7.2)**	**HCO_3^- buffer (pH 6.2)**	**PIPES buffer (pH 6.2)**
Time (hrs)	**pH**	**pH**	**pH**	**pH**
0	7.24 ± 0.35	7.22 ± 0.01	6.25 ± 0.04	6.27 ± 0.02
24	8.00 ± 0.01 †	7.29 ± 0.01	6.99 ± 0.04 †	6.31 ± 0.01
48	8.12 ± 0.03 †	7.28 ± 0.02	7.10 ± 0.11 †	6.31 ± 0.01
96	8.17 ± 0.04 †	7.28 ± 0.03	7.58 ± 0.09 †	6.34 ± 0.02

Table 3.1. Media pH of DMEM buffered with HEPES, PIPES, or HCO_3^- buffers over 96 hours in different O_2 tensions. *P=<0.05; †P=<0.01.

In order to assess the efficacy of the 14 day alginate culture period to restore or maintain the chondrocyte phenotype from cell isolation and monolayer expansion, markers of an articular chondrocyte phenotype in samples of cells (n=3 donors) were taken immediately after the alginate culture period, prior to the experimental time course (t=0). Each cell lysate was probed with primary antibodies against COL2A1 and COMP for identification and evaluation of a chondrocyte phenotype.

Figure **3.2** shows a representative image of COL2A1 and COMP protein expression from three separate donors. Western blot analysis confirmed the expression of COL2A1 and COMP and thus the presence of articular chondrocyte ECM proteins after 14 days in alginate bead culture in 5% O_2. This suggests the maintenance of a differentiated articular chondrocyte phenotype and the ability of these cells to synthesise ECM components within this 3-D culture system after 14 days incubation. Additionally, a primary antibody against caspase-3 was used to detect the presence of apoptosis in these samples. Caspase-3 was detected in cell lysates but expression was significantly lower (P=0.010) than the loading control, indicating low levels of apoptosis occurred during the culture period or on cell release from alginate bead culture (figure **3.2**).

Figure **3.3** demonstrates changes in media pH in response to varying O_2 tensions and IL-1β at pH 7.2 (normal pH) or pH 6.2 (acidosis). Media pH remained constant over 96 hours in all conditions studied (including when incubated with IL-1β), apart from when cultured in anoxia, where significant reductions in extracellular pH occurred within 24 hours to values around pH 5.8 (all P<0.05). After 96 hours, the pH of extracellular media was reduced to ~pH 5.5 in all anoxic conditions studied (all P=<0.05) (figure **3.3 A-D**). The effect in anoxic conditions was not altered by media pH (pH 7.2 or 6.2) or exposure to IL-1β. The effect in anoxia was related to the presence of chondrocytes as cell-free conditions did not show any alteration to media pH during identical incubation periods (see **Appendix i** (figure 1)).

Alginate encapsulated chondrocytes were then incubated at <1%, 2.5%, 5% and 21% O_2 for 24, 48 and 96 hours in acidic or normal pH conditions and in the

absence or presence of 10ng/ml IL-1β. The concentration of O_2 was measured in media samples and is shown in Table **3.2**. No differences in O_2 tension were found between normal, acidic or IL-1β treated conditions so data was pooled. O_2 levels at 2.5% and 5% remained steady-state. Despite the use of sodium dithionite, true anoxia was not achieved and mean O_2 levels of 0.7 ± 0.37% were recorded. Therefore, the term anoxia used in this study means <1% O_2. In hyperoxic O_2 conditions (21%), O_2 levels were reduced to 12.0 ± 0.6% at 24 hours, remaining at this level over the 96 hour incubation period. This finding may reflect alterations in chondrocyte physiology, such as increased oxygen consumption when exposed to an oxygen-rich environment and may suggest a metabolic shift from glycolysis to oxidative phosphorylation (Mignotte et al 1991).

Mean O_2 tension of media following incubation conditions				
	<1%	**2.5%**	**5%**	**21%**
24 Hours	1.2	2.6	5.6	12.7
48 Hours	0.9	2.2	4.9	10.8
96 Hours	0.14	2.8	5.07	12.6
Mean	**0.7% ± 0.37**	**2.5% ± 0.16**	**5.2% ± 0.22**	**12.0% ± 0.62**

Table 3.2. O_2 levels of media after experimental incubations. HOAC (passage 3) were cultured in alginate beads for 14 days and then incubated in <1%, 2.5%, 5% or 21% O_2 in normal (pH 7.2) or acidic (pH6.2) media in the absence or presence of 10ng/ml IL-1β for 0-96 hours. Aliquots of cell culture media were harvested immediately after experimental exposure and O_2 levels were estimated using a Hansatech oxygen electrode (following calibration). Results represent mean ± SEM O_2 levels after 24, 48 and 96 hours (n=3 donors).

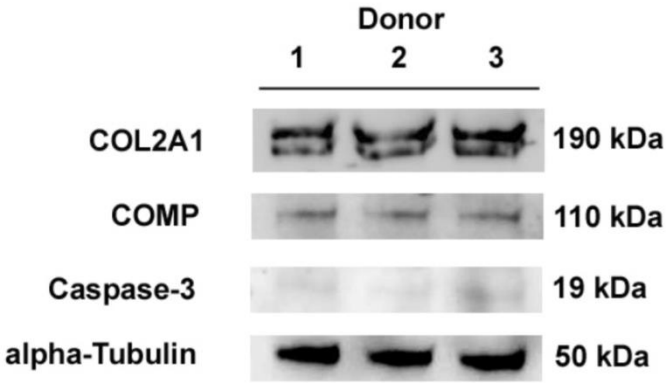
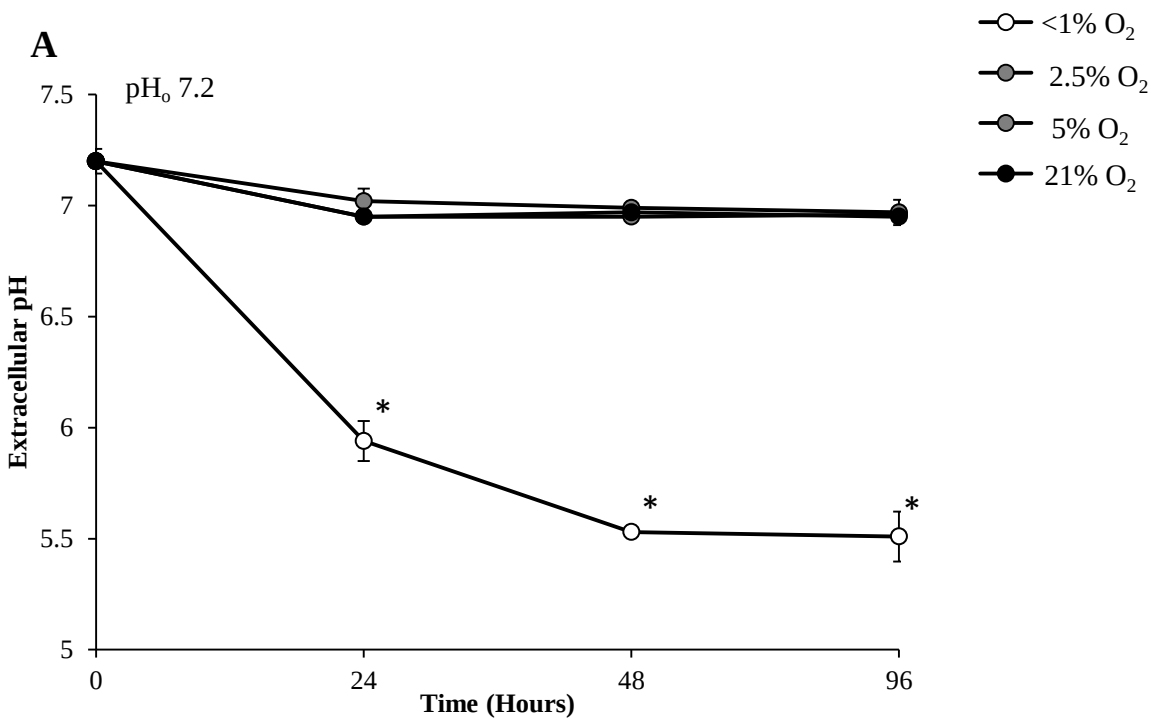
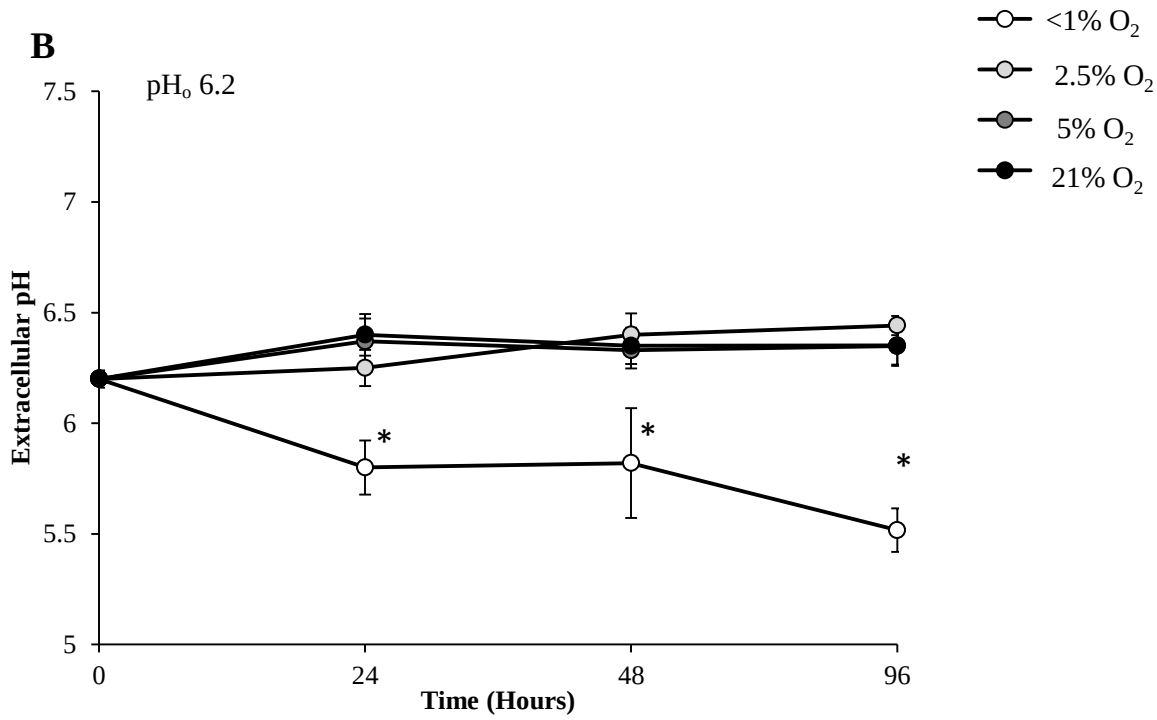


Figure 3.2. Expression of ECM components in t=0 chondrocytes lysates. 3 monolayer expanded chondrocytes were cultured in three-dimensional alginate microspheres for 14 days. Western blotting was performed on cell lysates taken on day 14 of the alginate culture period (t=0). The protein expression of three separate HOAC samples (n=3) probed with antibodies against collagen type II alpha 1 (COL2A1), cartilage oligomeric matrix protein (COMP) and caspase-3 (CASP-3) are shown. Alpha-tubulin was used as a loading control.


A
pHo 7.2
7.5
7
6.5
6
5.5
5
Extracellular pH
0
24
48
96
Time (Hours)
*
*
*
<1% O2
2.5% O2
5% O2
21% O2


B
pHo 6.2
7.5
7
6.5
6
5.5
5
Extracellular pH
0
24
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96
Time (Hours)
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<1% O2
2.5% O2
5% O2
21% O2

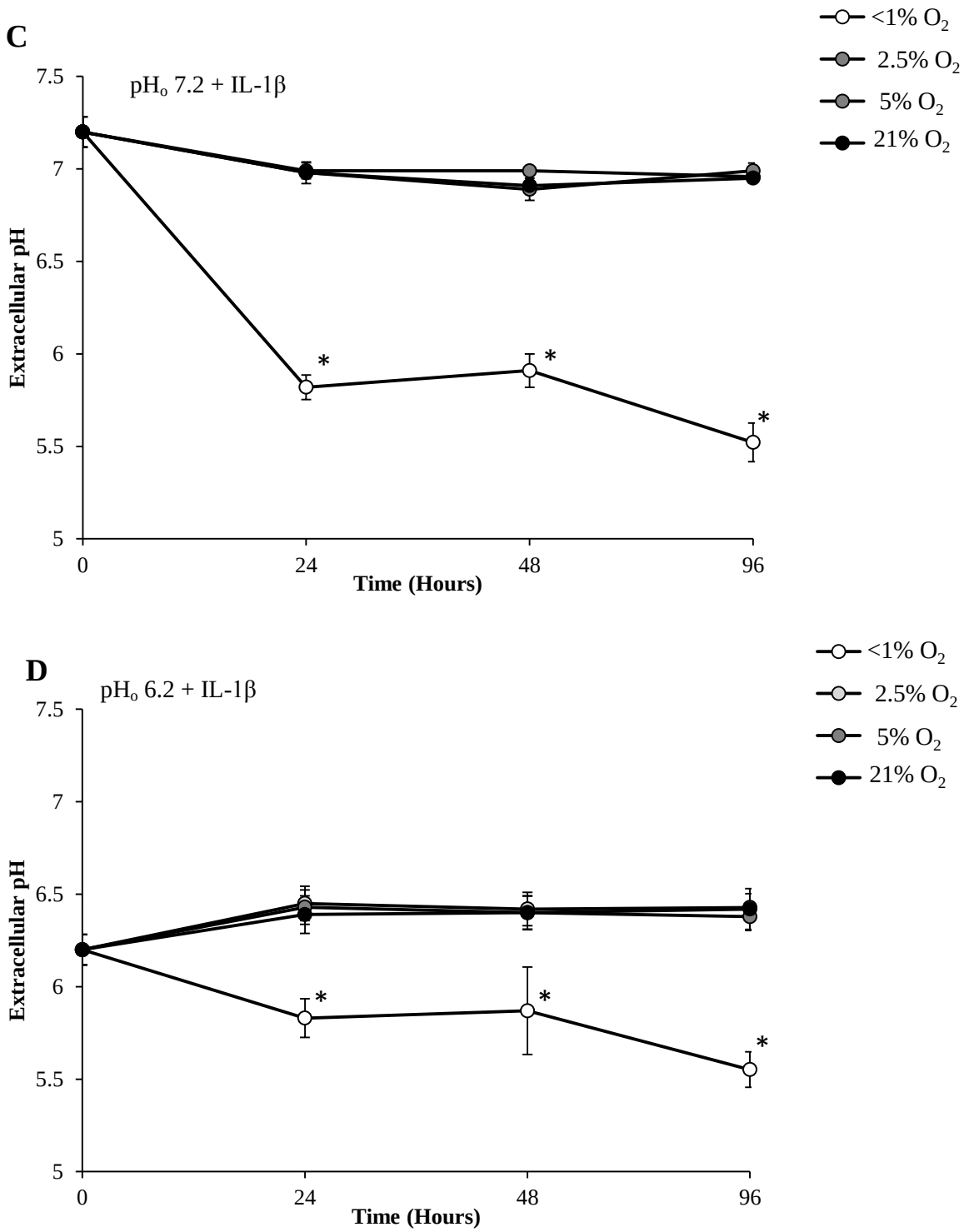


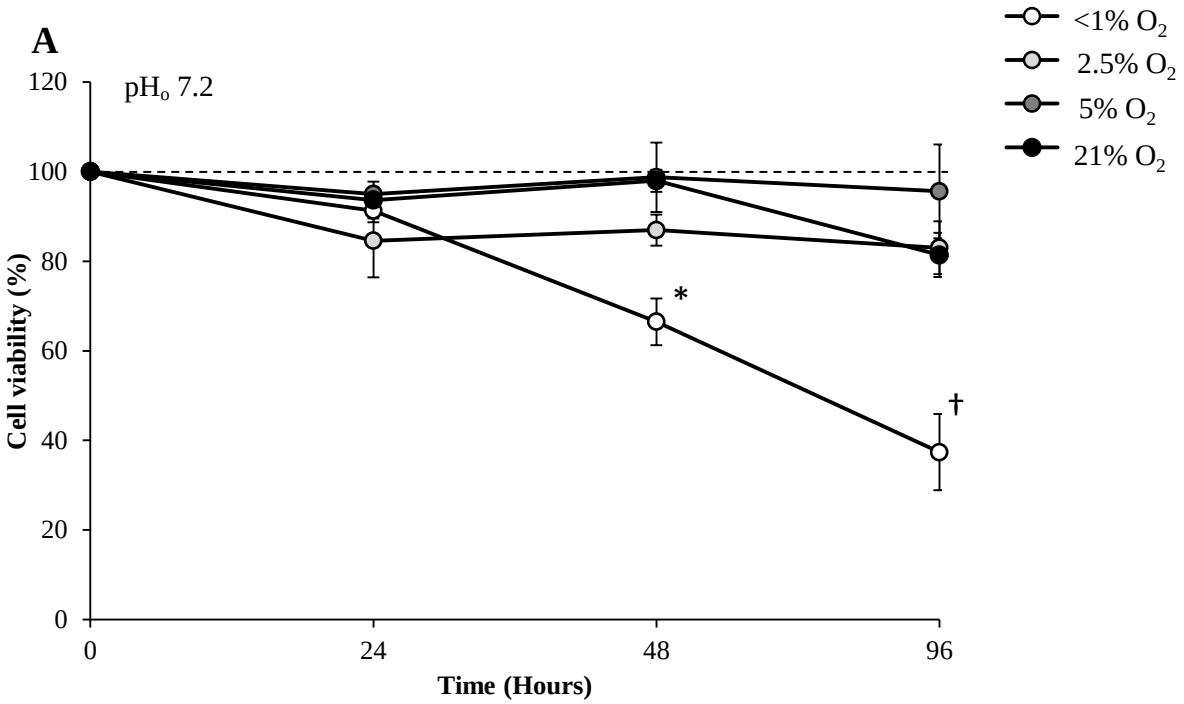
Figure 3.3. Effect of different O_2 levels and IL-1β on media pH. Passage 3 HOAC were cultured in alginate beads for 14 days and then incubated for 24, 48 or 96 hours in <1%, 2.5%, 5% or 21% O_2. Panels **A-D** show changes in extracellular pH of HOAC cultured in either normal (pH 7.2) or acidic (pH 6.2) media in the presence or absence of IL-1β. Results represent mean ± SEM pH values (n=3 donors). *P=<0.05 compared to control (time-0) (P=<0.05).

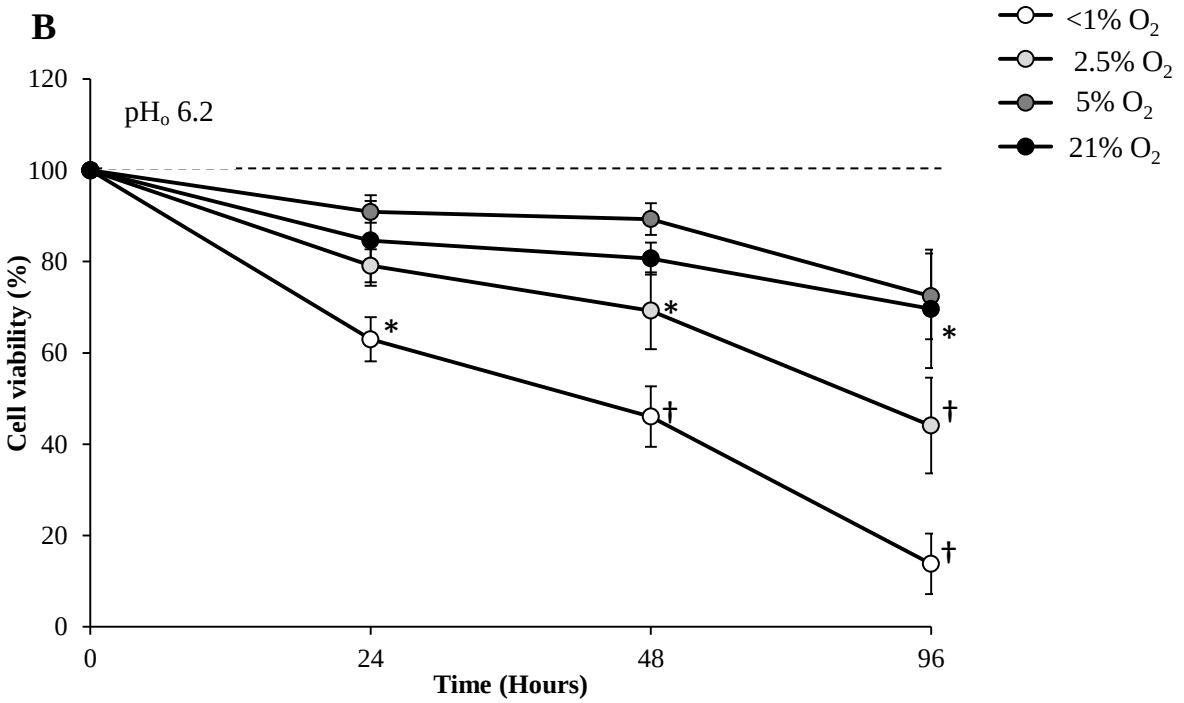
3.4.2. Chondrocyte cell viability

Cell viability and cell counts were assessed every 48 hours during the 14 day alginate culture period. No change in cell viability or cell count was observed over the culture period (see **Appendix i** (figure 2)). Figure **3.3** shows the effect of varying O_2 tensions, pH and IL-1β on chondrocyte cell viability after 24, 48 and 96 hours. The results show significant effects of O_2 tension and extracellular pH on human osteoarthritic chondrocyte cell viability, augmented in the presence of IL-1β.

As can be seen from figure **3.4 A**, cell viability at pH 7.2 did not significantly change when cells were cultured in 2.5%, 5% or 21% O_2 tensions over the 96 hour incubation period, but were significantly reduced by 96 hours in <1% O_2 tensions compared to control (37.4 ± 8.5%, $P<0.001$). In acidosis (figure **3.4 B**), cell viability was maintained at values comparable to control when cultured in 5% O_2 over 96 hours. Cell viability was maintained in hypoxia (2.5% O_2) and hyperoxia (21% O_2) at 24 hours but reduced significantly at 48 hours in hypoxia (69.2% ± 8.4%, $P=0.03$) and at 96 hours in hyperoxia (69.6 ± 13.0%, $P=0.01$) respectively. Culture in anoxia (<1% O_2) led to significant reductions in cell viability at 24 hours (63.0 ± 4.8%, n=3, $P=0.04$), which by 96 hours was reduced to 13.8 ± 6.6% of control values ($P<0.001$).

The presence of IL-1β significantly reduced the viability of osteoarthritic chondrocytes in all O_2 tensions studied. In pH 7.2 conditions, at 96 hours, cell viability was reduced to 3.1 ± 1.9%, 44.8 ± 7.2%, 62.9 ± 5.5% and 20.1 ± 4.9% (all $P=<0.05$) at <1%, 2.5%, 5% and 21% O_2 tensions respectively (figure **3.4 C**). Of specific note is the observation that cell viability at 5% O_2 is significantly higher than 21% O_2 after 96 hours in the presence of IL-1β (63.0 ± 5.5% versus 20.1 ± 4.9%, $P=0.001$). Acidosis and the addition of IL-1β led to the most pronounced reductions in cell viability after 24 hours in this study ($P=<0.05$). Cell viability was further reduced at 48 and 96 hours at all oxygen tensions studied (figure **3.4 D**).


A
pHo 7.2
<1% O2
2.5% O2
5% O2
21% O2
Cell viability (%)
Time (Hours)
0
20
40
60
80
100
120
0
24
48
96
*
†


B
pHo 6.2
<1% O2
2.5% O2
5% O2
21% O2
Cell viability (%)
Time (Hours)
0
20
40
60
80
100
120
0
24
48
96
*
†

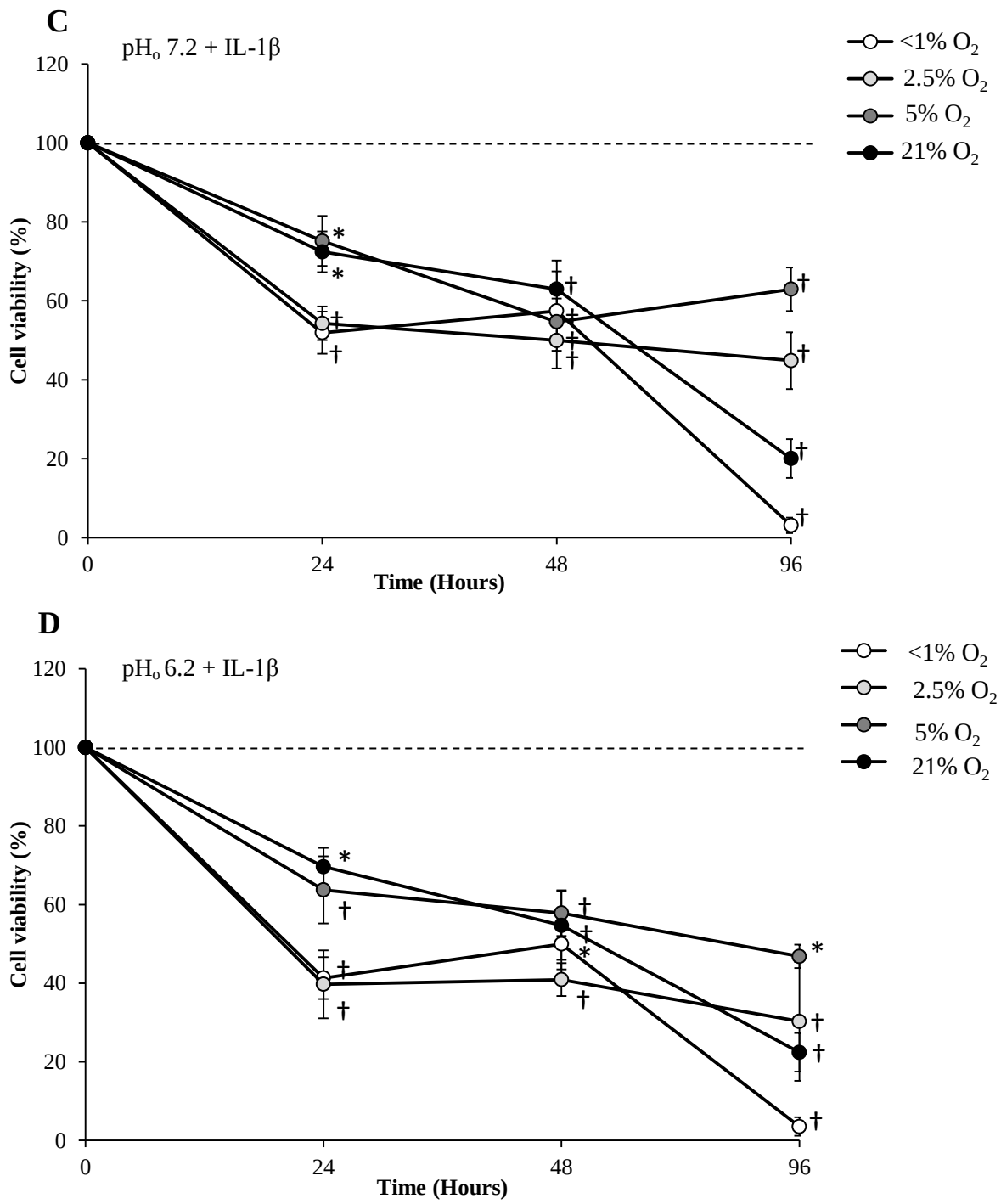
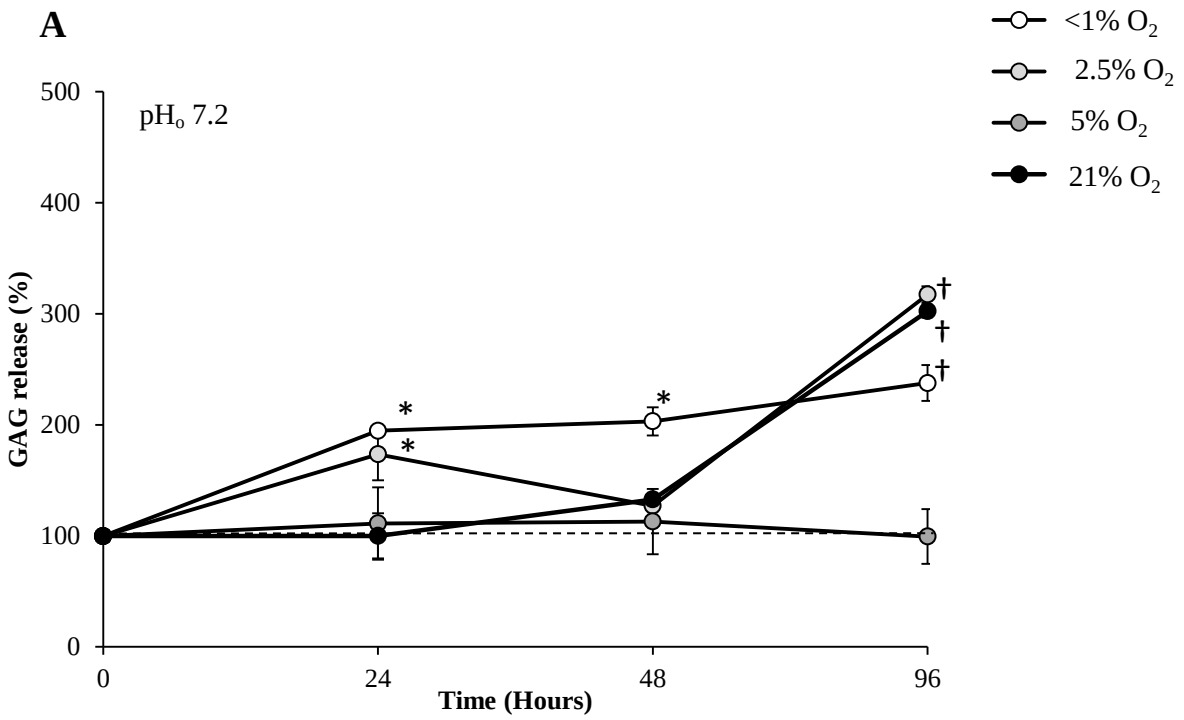


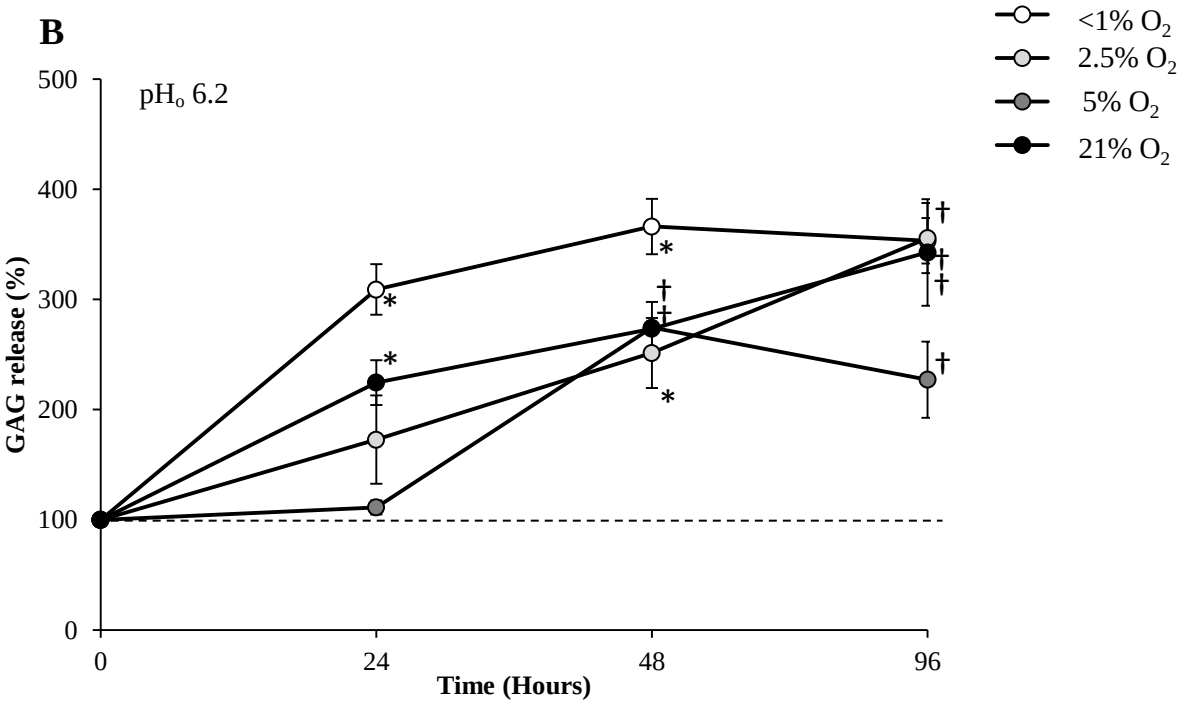
Figure 3.4. Effect of oxygen tension, pH and IL-1β on cell viability of HOAC over 96 hours. Cell viability was determined by the trypan blue exclusion and presented as mean values compared to control (time=0, 5% O_2, pH7.2). Osteoarthritic chondrocytes were cultured in three dimensional alginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia for chondrocytes) or 21% O_2 (hyperoxia for chondrocytes) at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) and cell viability was measured after 24, 48 hours and 96 hours. Line graphs represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

3.4.3. Chondrocyte glycosaminoglycan release

The release of GAG rich degradation products into cell culture media is an early event reflecting proteoglycan degradation *in vitro* (Mort and Roughley, 2007). GAG release into media did not significantly alter over the 96 hour incubation period when cells where cultured in 5% O_2 and pH 7.2 conditions (99.5 ± 24.7% versus control, 100.8% ± 5.3%, n.s) (Figure **3.5A**). Similarly, GAG release in hypoxia (2.5% O_2) and hyperoxia (21% O_2) did not significantly alter from control values at 48 hours, but at 96 hours, were increased three-fold compared to control. Culture of chondrocytes in anoxia (<1% O_2) showed significant increases in GAG release at all time points compared to control (figure **3.5 A**) and were significantly different to values at 5% O_2, pH 7.2, t=48 hours (see table **3.3**). In addition, acidosis significantly increased GAG release (figure **3.5 B**). GAG release was ~3.5 fold higher after 96 hours in all O_2 tensions studied, with the exception of 5% O_2, which increased by 127% (P=0.007).

In the presence of IL-1β, in pH 7.2 conditions, GAG release was significantly increased by ~4.5 fold in every O_2 tension studied (all P<0.05) (figure **3.5 C**). After 48 and 96 hours, GAG release was reduced but was still significantly different from control in all O_2 tensions (all P<0.05). This latter finding may reflect GAG depletion later in the time-course. Similar to pH 7.2 conditions, culture of HOAC in acidosis (pH 6.2) and IL-1β significantly increased GAG release by 3-4 fold compared to control at 96 hours (all P<0.05) (figure **3.5 D**).


A
pHo 7.2
<1% O2
2.5% O2
5% O2
21% O2
GAG release (%)
500
400
300
200
100
0
0
24
48
96
Time (Hours)
*
*
*
†
†
†


B
pHo 6.2
<1% O2
2.5% O2
5% O2
21% O2
GAG release (%)
500
400
300
200
100
0
0
24
48
96
Time (Hours)
*
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*
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†

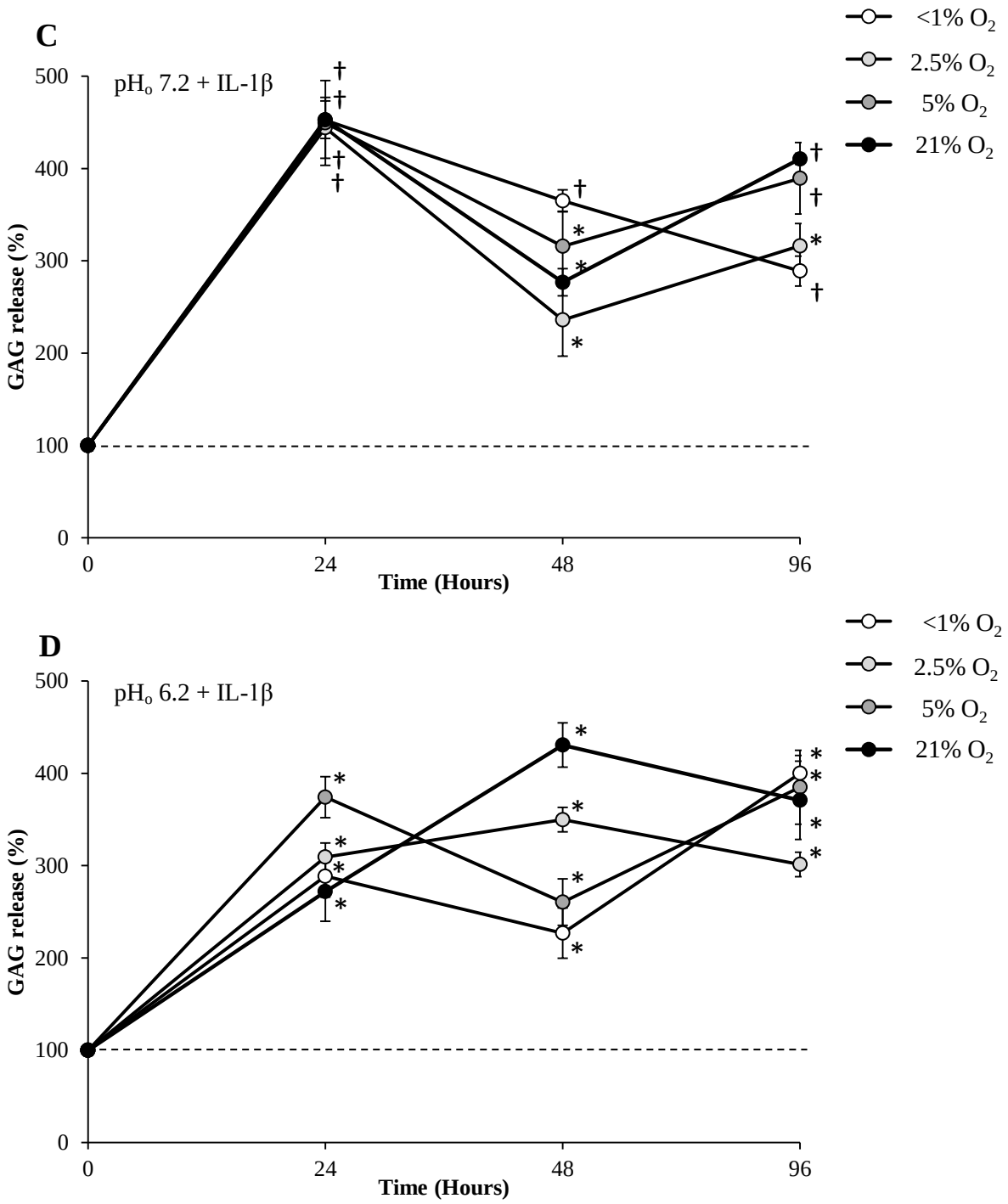


Figure 3.5. Effect of oxygen tension, pH and IL-1β on HOAC GAG release over 96 hours. GAG release was determined by the DMMB method and presented as mean values compared to control (time=0, 5%O_2, pH7.2). OA chondrocytes were cultured in 3-D alginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia) or 21% O_2 (hyperoxia for chondrocytes) at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) and GAG release was measured after 24, 48 hours and 96 hours. Line graphs represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

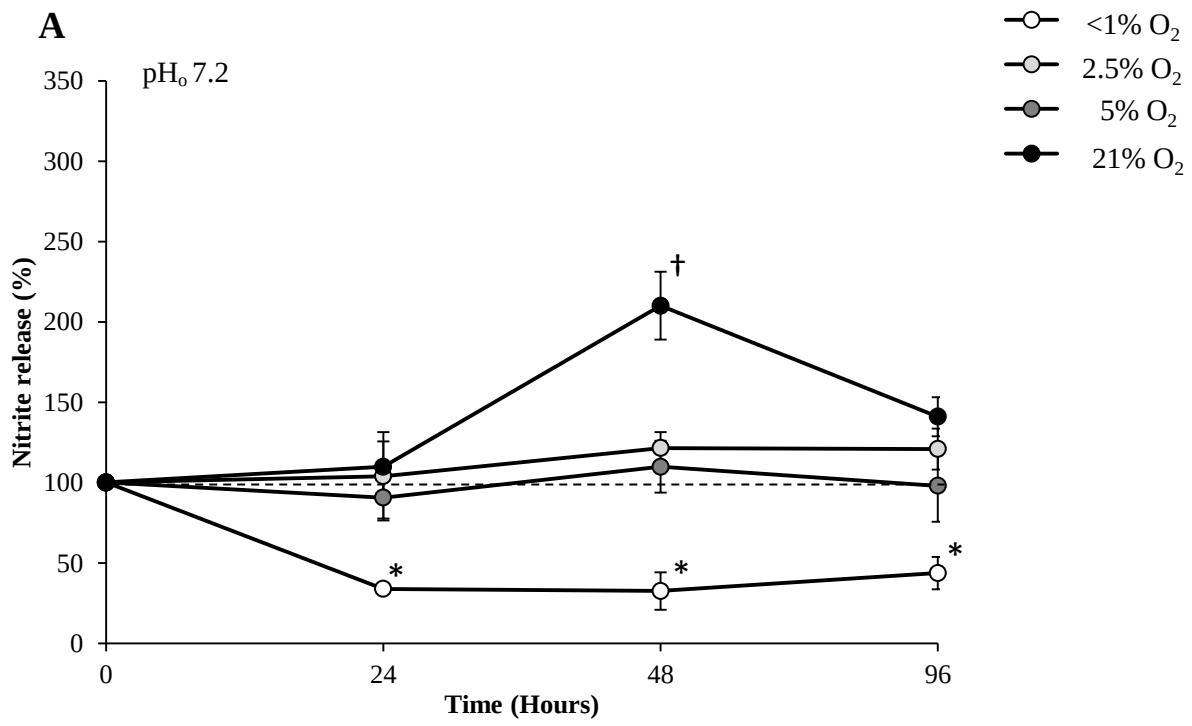
3.4.4. Chondrocyte nitric oxide production

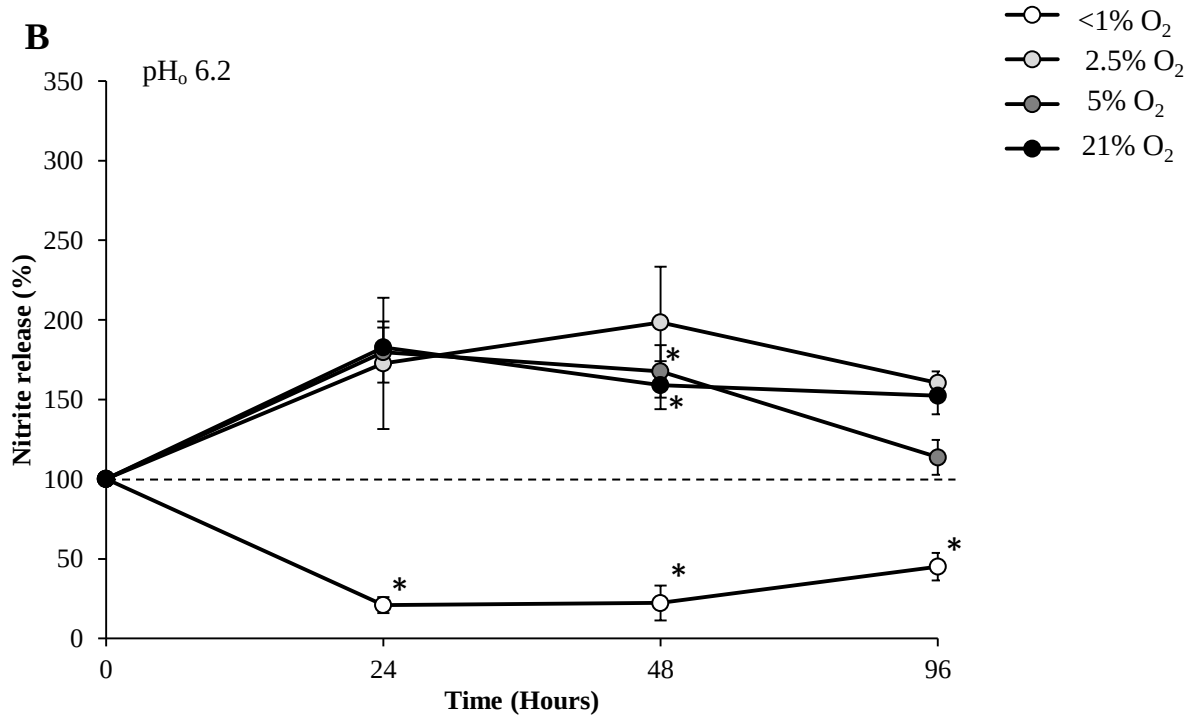
The Griess reaction was used to detect for nitrite and nitrate release into culture media as a marker of nitric oxide (NO) production. NO production is a molecular mediator of many physiological processes and its overproduction correlates with inflammation and oxidative stress, being implicated in the pathogenesis of OA. The synthesis of NO is largely O_2 dependent and therefore the effects of varying oxygen tensions on nitrite and nitrate production over 96 hours was studied. Additionally, the effect of acidosis and IL-1β to stimulate NO production over this time course was analysed.

HOAC cultured in anoxia (<1% O_2) displayed significantly reduced levels of detectable nitrite and nitrate release in cell culture medium indicating low NO production. This effect was independent of media pH or cytokine exposure and was evident at every time point studied which likely reflects the lack of available substrate (O_2) for NO synthesis (figure **3.6** and **3.7**). In agreement with the cell viability and GAG release data, nitrite and nitrate release did not significantly differ from control when chondrocytes were cultured in 5% O_2, pH 7.2 (figure **3.6 A**). Culture of chondrocytes in hyperoxia (21% O_2) however, led to a doubling in nitrite release at 48 hours compared to control (210.2 ± 21.1%, P=0.01) and compared to 5% O_2, t=48 hours (table **3.3**).

Acidosis significantly increased nitrite release at 5% and 21% O_2 at 48 hours but by 96 hours, values had returned to values comparable to control (figure **3.6 B**). Nitrate release in acidosis did not significantly change from control values at all-time points at 5 and 21% O_2 (figure **3.7 B**); however in acidosis and hypoxia (2.5% O_2, pH6.2) nitrate release was significantly increased at 24 and 48 hours. In the presence of IL-1β (5% O_2, pH 7.2), nitrite release was significantly increased at 24 and 48 hours (202.6 ± 26.1% and 170.8 ± 1.3% respectively, both P=<0.05) but decreased to values comparable to control at 96 hours (figure **3.6 C**). Similarly, in hypoxia (2.5%) and hyperoxia (21% O_2), nitrite release was significantly increased at 48 hours (253.2 ± 41.8% and 185.1 ± 21.6% respectively, both P=<0.05). After 96 hours,

nitrite release returned to values comparable to control in hyperoxia (167.2 ± 45.0%) but remained significantly elevated in hypoxia (294.7 ± 7.2%) (figure **3.6 C**). The addition of IL-1β to acidotic media did not significantly alter nitrite or nitrate release apart from in 2.5% O_2 conditions (figures **3.6 D and 3.7 D**).


A
pHo 7.2
<1% O2
2.5% O2
5% O2
21% O2
Nitrite release (%)
350
300
250
200
150
100
50
0
0
24
48
96
Time (Hours)
†
*
*
*


B
pHo 6.2
<1% O2
2.5% O2
5% O2
21% O2
Nitrite release (%)
350
300
250
200
150
100
50
0
0
24
48
96
Time (Hours)
*
*
*
*
*

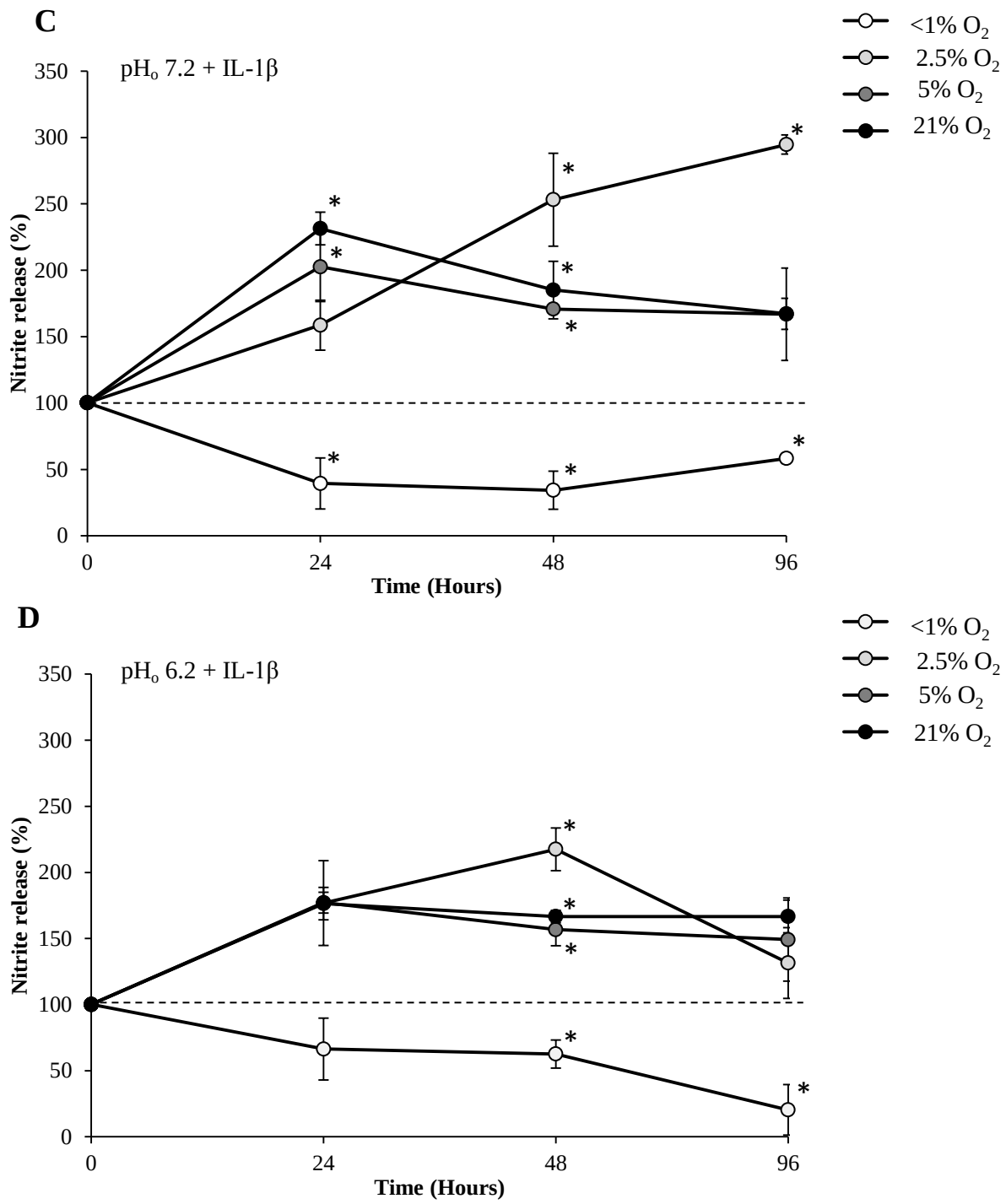
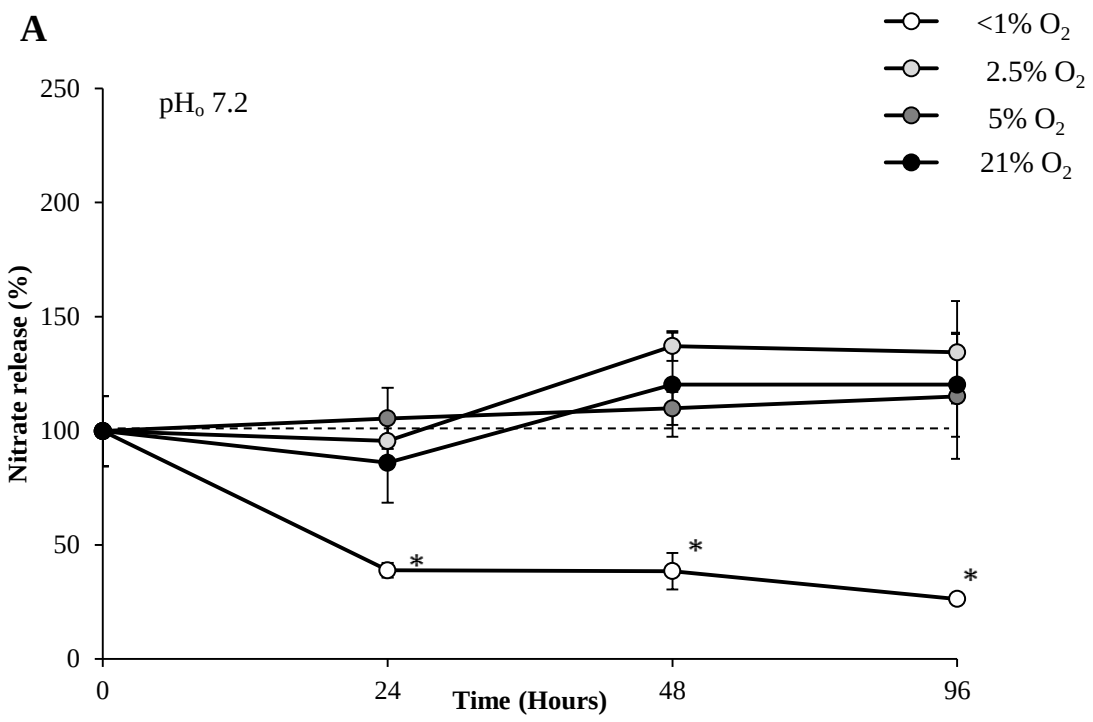
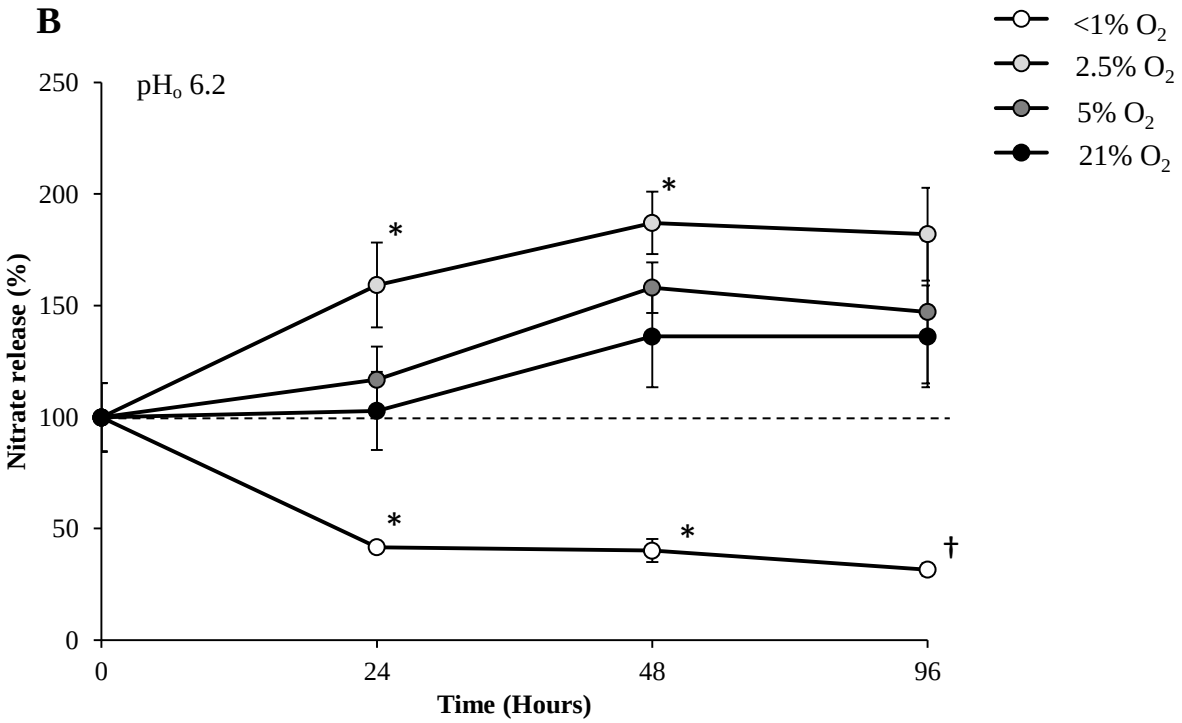


Figure 3.6. Effect of oxygen tension, pH and IL-1β on nitrite release over 96 hours. Nitrite release was determined by the Griess assay and presented as mean values compared to control (time=0, 5%O_2, pH7.2). OA chondrocytes were cultured in 3-Dalginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia) or 21% O_2 (hyperoxia for chondrocytes) at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) and nitrite release was measured after 24, 48 hours and 96 hours. Line graphs represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.


A
pHo 7.2
<1% O2
2.5% O2
5% O2
21% O2
Nitrate release (%)
250
200
150
100
50
0
0
24
48
96
Time (Hours)
*
*
*


B
pHo 6.2
<1% O2
2.5% O2
5% O2
21% O2
Nitrate release (%)
250
200
150
100
50
0
0
24
48
96
Time (Hours)
*
*
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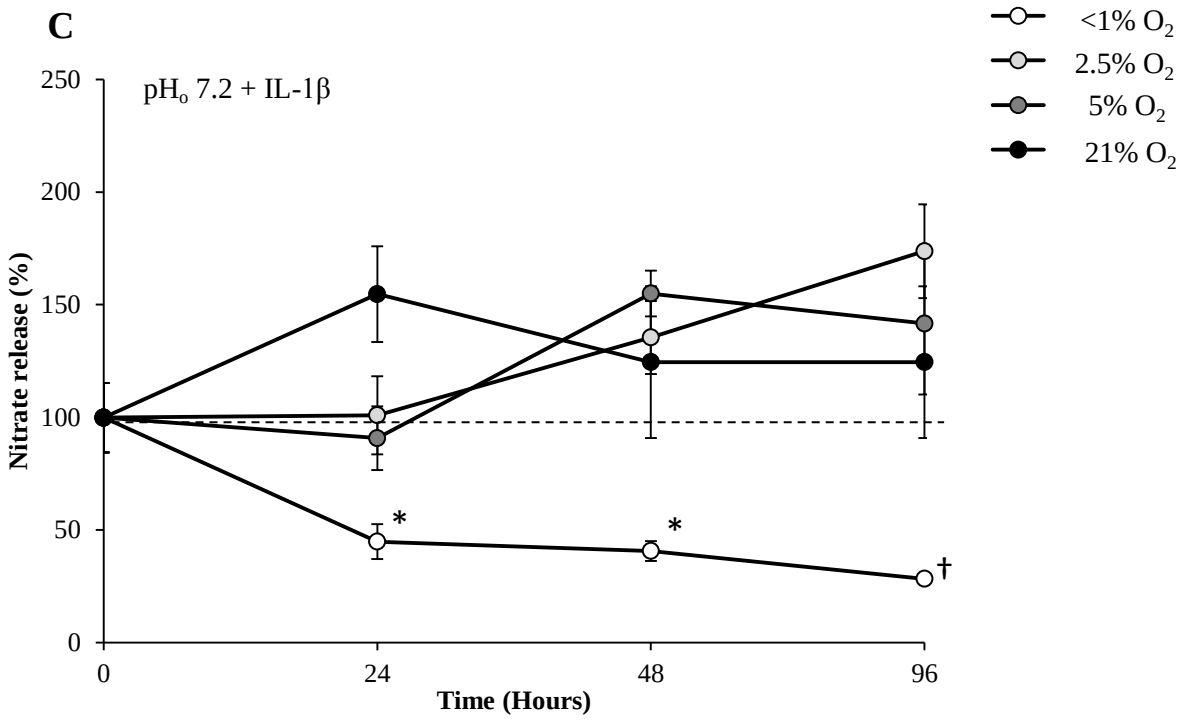


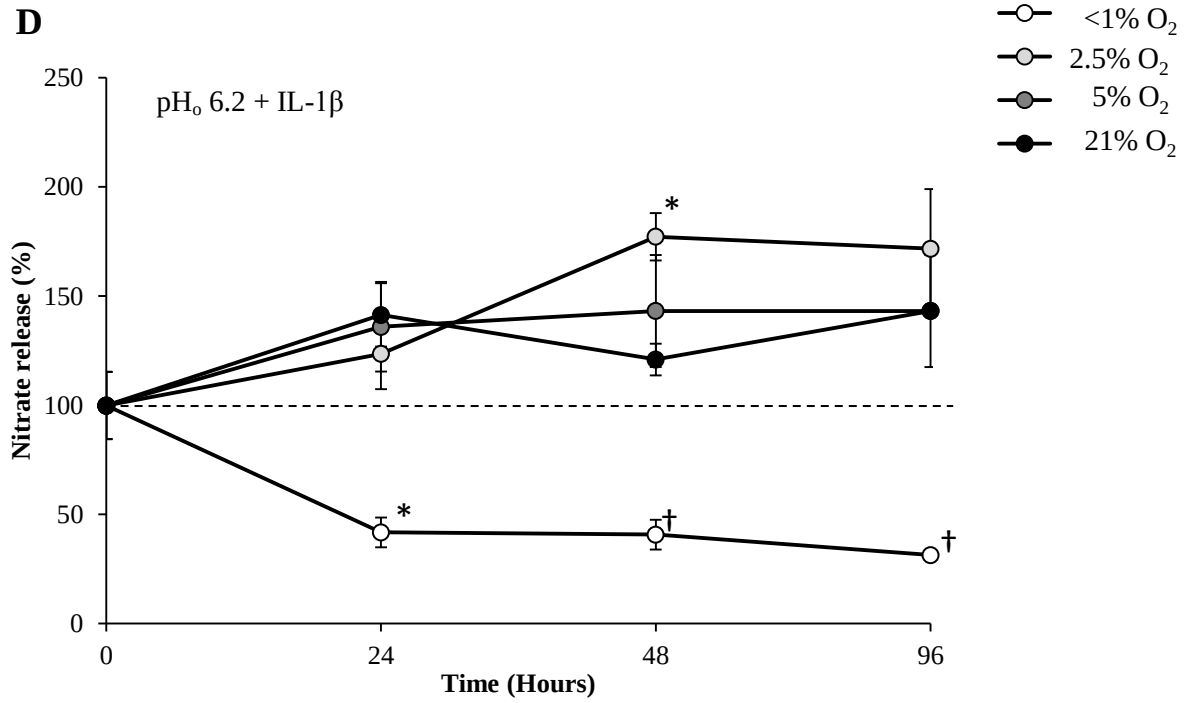


Figure 3.7. Effect of oxygen tension, pH and IL-1β on nitrate release over 96 hours. Nitrate release was determined by the Griess assay and presented as mean values compared to control (time=0). Human OA articular chondrocytes were cultured in 3-D alginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia) or 21% O2 (hyperoxia) at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) and nitrate release was measured after 24, 48 hours and 96 hours. Line graphs represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

3.4.5. Intracellular ATP (ATP_i) measurement

The generation of ATP supports chondrocyte ECM synthesis. ATP depletion as a result of altered respiratory activity and metabolism may be altered during the progression of osteoarthritis which could compromise ECM integrity (Johnson et al, 2004). To determine the effect of varying oxygen tensions and extracellular pH in osteoarthritic chondrocytes, intracellular ATP was measured over 96 hours in chondrocytes exposed to <1, 2.5, 5 or 21% O_2 tensions in either normal (pH 7.2) or acidic (pH 6.2) media.

Intracellular ATP (ATP_i) levels at 5% O_2 were unchanged across the time course (4.0 ± 0.2µM at 96 hours) versus control (4.0 ± 0.3µM). Culture in 21% O_2 increased ATP_i production compared to 5% O_2, although this was not statistically significant from control. In pH 7.2 media and hypoxic and anoxic conditions, ATP_i was significantly reduced after 24 hours and remained reduced over the time course (figure **3.8 A).** Culture in acidosis led to significant reductions in ATP_i at all oxygen concentrations (figure **3.8 B**).

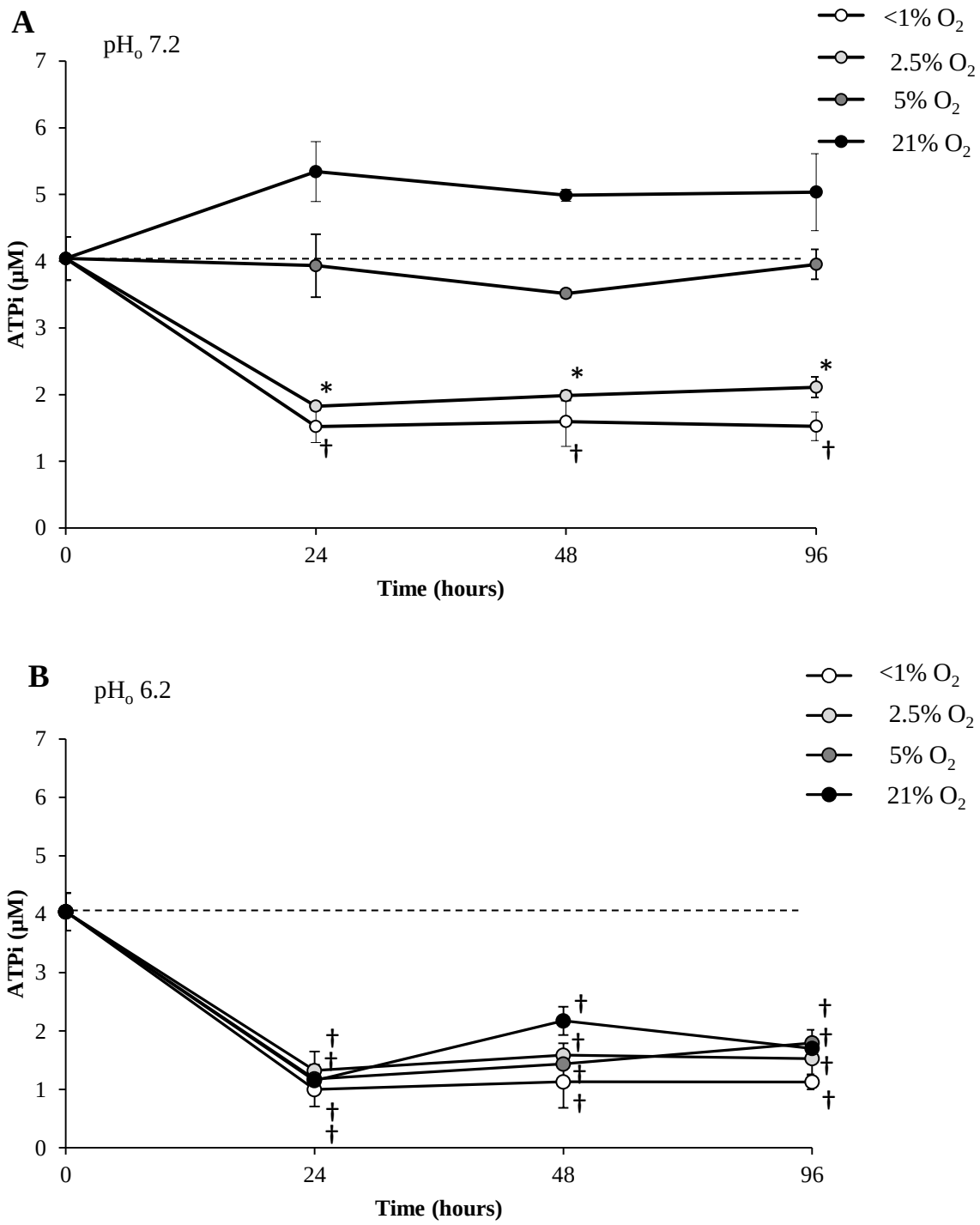


Figure 3.8. Effect of oxygen tension and pH on intracellular ATP (ATP_i) in human osteoarthritic articular chondrocytes over 96 hours. Osteoarthritic chondrocytes were cultured in three dimensional alginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia for chondrocytes) or 21% O_2 (hyperoxia for chondrocytes) at pH 7.2 (normal pH) (**A**) or 6.2 (acidosis) (**B**) and ATP_i was determined after 24, 48 hours and 96 hours using the EnzyLightTM ATP assay kit. Line graphs represent means ± SEM, n=3 donors. Results are presented to control (time=0, 5% O_2, pH7.2). *P=<0.05; †P=<0.01 compared to control.

3.4.6. Measurement of total MMP-13

MMP-13 plays a significant role in the osteoarthritic disease process, stimulating degradation of the cartilage matrix and inducing cellular apoptosis and mitochondrial dysfunction (Lopez-Armada et al, 2006) potentially through redox sensitive (ROS/NO) pathways (Yasuhara et al, 2005).

The results of this study showed that the release of MMP-13 was not influenced by oxygen tension or pH after 96 hours incubation (Fig **3.9**). An oxygen tension of 5% in pH 7.2 conditions (suggested optimal chondrocyte environmental conditions) resulted in the lowest MMP-13 release (143 pg/ml) after 96 hours.

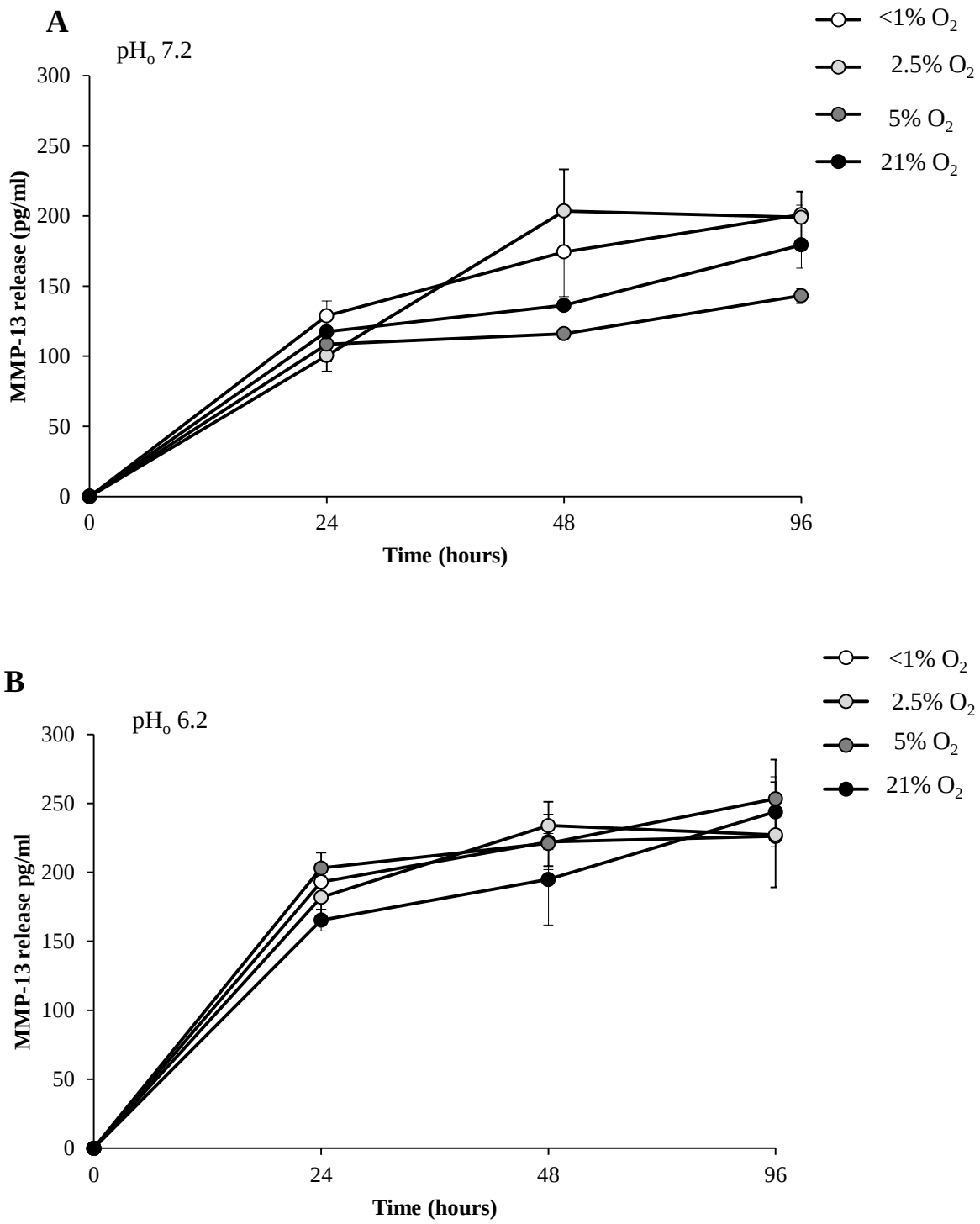


Figure 3.9. Effect of oxygen tension and pH on MMP-13 release from human osteoarthritic articular chondrocytes over 96 hours. Osteoarthritic chondrocytes were cultured in three dimensional alginate beads in <1% (anoxia), 2.5% (hypoxia), 5% (normoxia for chondrocytes) or 21% O_2 (hyperoxia for chondrocytes) at pH 7.2 (normal pH) (**A**) or 6.2 (acidosis) (**B**) and MMP-13 release was determined after 24, 48 hours and 96 hours using an active MMP-13 ELISA. Line graphs represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

Treatment			Cell viability		GAG release		Nitrite release		Nitrate release	
O_2 (%)	pH_o	IL-1β	(%)	*P* value	(%)	*P* value	(%)	*P* value	(%)	*P* value
5 (48hrs)	**7.2**	-	98.7± 7.7	**N/A**	109. 9 ± 2.0	**N/A**	109.9 ± 16.3	**N/A**	109.7 ± 7.1	**N/A**
<1	**7.2**	-	66.4 ± 5.42	0.009*	203.1 ± 12.6	0.018*	32.6 ± 11.6	0.018*	38.4 ± 7.9	0.007*
2		-	86.9 ± 3.5	0.444	127.0 ± 4.9	0.862	121.6 ± 9.9	0.948	137.0 ± 6.5	0.353
5		-	98.7 ± 7.7	1.000	109.9 ± 2.0	1.000	109.9 ± 16.3	1.000	109.7 ± 7.1	1.000
21		-	97.9 ± 2.4	1.000	132.8 ± 21.0	0.740	210.1 ± 21.0	0.003*	120.1 ± 22.8	0.917
<1	**6.2**	-	46.0 ± 15.4	0.006*	366.2 ± 25.1	0.000†	22.3 ± 10.9	0.041*	40.1 ± 5.1	0.042*
2		-	69.2 ± 8.4	0.120	251.4 ± 31.8	0.009*	198.4 ± 34.9	0.038*	187.0 ± 14.0	0.064
5		-	89.3 ± 3.5	0.865	274.4 ± 23.2	0.003*	167. ± 16.6	0.205	157.9 ± 11.3	0.312
21		-	84.5 ± 3.5	0.639	273.4 ± 4.6	0.003*	159.0 ± 15.1	0.316	136.1 ± 40.1	0.754
<1	**7.2**	+	57.4 ± 10.0	0.012*	365.0 ± 11.8	0.000†	34.3 ± 14.3	0.005*	40.6 ± 4.3	0.044*
2		+	49.9 ± 7.0	0.004*	235.9 ± 39.2	0.042*	253.1 ± 41.8	0.005*	135.4 ± 16.1	0.693
5		+	54.6 ± 5.8	0.008*	315.7 ± 37.9	0.002*	170.8 ± 1.3	0.250	154.9 ± 10.1	0.270
21		+	62.9 ± 7.2	0.027*	276.7 ± 14.7	0.008*	185.1 ± 21.6	0.129	124.4 ± 33.7	0.929
<1	**6.2**	+	49.9 ± 6.4	0.002*	226.8 ± 27.2	0.026*	48.1 ± 2.1	0.019*	40.7 ± 6.8	0.007*
2		+	40.9 ± 4.1	0.000†	349.8 ± 13.3	0.000†	217.5 ± 40.1	0.048*	177. 1± 10.8	0.021*
5		+	46.8 ± 5.8	0.001*	260.4 ± 25.1	0.006*	156.8 ± 12.3	0.546	143.1 ± 25.5	0.334
21		+	54.6 ± 8.7	0.003*	430.6 ± 24.0	0.000†	166.6 ± 4.6	0.394	120.9 ± 45.6	0.585

Table 3.3. Statistical analysis comparing raw data at t=48 with values at pH 7.2, 5% O_2, t=48. Chondrocyte cell function appeared to be significantly altered altered at 48 hours compared to t=0 (pH 7.2, 5% O_2). Therefore statistical analysis was performed to see assess if values at t=48 were significantly altered compared to values at pH 7.2, 5% O_2 t=48. *P=<0.05; †P=<0.01.

3.5. Discussion

Articular cartilage is a specialised tissue covering the surface of joints. Cartilage provides a unique environment for its resident cell, the chondrocyte, which during joint disease is significantly altered. The precise aetiopathogenesis of OA is not fully elucidated, but it is regarded as an imbalance between normal synthesis and degradation processes within the articular joint tissue with an insufficient capacity of tissues to repair subsequent damage (Blanco et al, 2004; Chen et al, 2006; Sandell & Aigner, 2001). Despite being a multifactorial arthropathy, it is widely accepted that environmental homeostasis is considerably altered in OA cartilage, which ultimately compromises the integrity of the chondrocyte and its matrix. O_2 tensions and pH levels represent key environmental parameters that are altered during OA progression and form the focus of this project.

Cell culture analysis and considerations

This chapter details the development of a three dimensional *in vitro* chondrocyte model, revolving around varying O_2 tensions and pH, aiming to mimic a range of (patho) physiological conditions present in articular joints. Here, the important interactions and effects of varying O_2 tensions and pH on human osteoarthritic articular chondrocytes are demonstrated. In this study, human osteoarthritic chondrocytes (HOACs) were sensitive to low levels of O_2 and acidosis which represent conditions that occur in the diseased joint (Biniecka et al 2010; Konttinen et al, 2002; Levick, 1990; Mapp et al, 1995). This work also underlines the effect of culture conditions on chondrocyte activity and demonstrates that studying chondrocyte biology at non-physiological levels of oxygen may be inappropriate. Although the effect of oxygen tensions and pH *alone* on chondrocyte phenotype and matrix synthesis is well documented, this is the first investigation detailing the interactive effects of oxygen and pH on chondrocyte function over such a broad range of environmental parameters.

Chondrocytes were also exposed to IL-1β to assess the effects of this pro-inflammatory cytokine on chondrocyte function under these different oxygen and pH

levels. IL-1β exacerbated responses observed in IL-1β-free conditions (on cell viability and GAG release) and augmented nitrite release, particularly in hypoxia and physiological pH.

For the purposes of this study, human articular chondrocytes were used from osteoarthritic joints since the primary aim was to evaluate the response of diseased tissue to alterations in the extracellular environment (pH and O_2) over a defined time course. Comparison to non-diseased human tissue would have been advantageous to determine differences in cell responses to alterations in the physicochemical environment, but this was not achievable due to a limited supply of 'normal' human articular chondrocytes. Direct comparison is complicated however, due to inherent heterogeneity of both normal and diseased tissue and the difficulty in obtaining age-matched controls.

This study adopted a 3-D alginate culture system as opposed to using monolayer culture. It is widely accepted that chondrocyte dedifferentiation occurs in monolayer culture with loss of collagen type II expression (Lin et al 2008) and that a 3-D culture environment allows for maintenance of a chondrocytic phenotype. Initially, chondrocytes were expanded in monolayer culture, to achieve appropriate cell densities, before incubation in a physiological O_2 tension (5% O_2) for 14 days in 3-D alginate beads. This incubation period was chosen based on previous published data indicating that alginate encapsulated chondrocytes incubated for 2 weeks at 5% O_2 displayed GAG deposition profiles and COL2A1 expression comparable to unpassaged chondrocytes (Murphy and Polack, 2007; Schneider et al, 2004). In agreement, results from this study show expression of the ECM components COL2A1 and COMP after 14 days in 3-D alginate beads which suggest maintenance of a chondrocyte phenotype despite initial monolayer expansion.

It is crucial to consider the effects of culture conditions on chondrocyte activity at every level. The composition of cell culture medium can affect chondrocyte behaviour and therefore is an important consideration for *in vitro* studies (van Osch et al, 2002). The goal of cell culture media is to mimic mammalian body fluid as closely as possible and maintain pH at a steady rate (Xu et al, 2006). Dulbecco's

Modified Eagles Medium (DMEM) is the preferred media choice in many laboratories as the salts and solutes contained therein are close to physiological levels (Xu et al, 2006). The production of lactate, especially by a predominantly anaerobic cell such as the chondrocyte, however, is a metabolic process capable of modifying extracellular pH (Xu et al, 2006). As such, the correct choice of buffer within the correct growth medium, to maintain pH at the level required, is a critical consideration for the culture of specific cell types.

Unlike other tissues, articular cartilage is unique in that the bicarbonate (HCO_3^-) buffering system plays little or no role in pH homeostasis *in vivo* and Na^+/H^+ exchange (NHE) predominates (Simpkin et al, 2007; Wilkins & Hall, 1992). Optimal functioning of NHE is critical for chondrocyte homeostasis (mutant cells lacking efficient NHE exchange are hypersensitive to acidosis). Essentially, the NHE is a Na^+ dependent membrane protein which importantly controls ion fluxes by exchanging one extracellular sodium ion for one intracellular proton in an electrochemically neutral fashion (Malo & Fliegel, 2006; Putney et al, 2002). Despite this, the vast majority of chondrocyte cell culture investigations utilise DMEM media supplemented with HCO_3^- leading to inappropriate alkalinisation (as seen in Table **3.1**) which is often unappreciated. To reduce this effect, HEPES (for media titrated to pH 7.2) and PIPES (for media titrated to pH 6.2) buffers were utilised in these investigations in order to activate the NHE. In the current study we demonstrate that HEPES and PIPES buffers maintain media pH at desired levels (pH 7.2 and pH 6.2 respectively) over 96 hours under varying oxygen tensions. HCO_3^- buffered media failed to maintain media pH and media alkalinisation occurred within 24 hours in every conditions studied. As a result, exogenous addition of HEPES and PIPES buffers where used in HCO_3^- free media throughout this project.

The effect of oxygen tension on OA chondrocyte function

The importance of oxygen as a regulator of chondrocyte activity has gained increasing recognition (Gibson et al 2008). Due to the avascularity of articular cartilage and relatively low levels of oxygen in the synovial fluid, oxygen tensions within cartilage are low and exhibit depth dependency (Zhou et al, 2004). Cartilage

oxygenation and cellular metabolism, cytokine stimulation, responses to hypoxia inducible factors and ultimately chondrocyte phenotype are all modified by O_2, and further reductions in O_2 tension from the norm are evident in OA (Grimshaw & Mason, 2000; Svalagosta & Kiaer, 1989).

The effects of 'hypoxia' on chondrocyte function can be difficult to determine in the literature due to the variable use of the terms 'hypoxia' and 'anoxia' in cartilage studies. In this study, <1% O_2 was used as anoxia and 2.5% O_2 as hypoxia since 'normoxia' for chondrocytes *in vivo* is around 5% O_2 (Zhou et al, 2004). Ambient oxygen tensions (21%) were therefore deemed hyperoxic in this study. *In vitro* culture of chondrocytes in ambient oxygen levels (21%) is common, but may be inappropriate. Work from others shows altered chondrocyte cell viability (Schneider et al, 2004), cellular morphology, ETC expression (Boubriak et al, 2009), metabolism and increased oxidative DNA damage (Heywood and Lee, 2010) in hyperoxia. Therefore the current study set out to assess the effects of abnormally high and low oxygen tensions on chondrocyte function.

In this study, culturing chondrocytes under 5% O_2 conditions appeared to result in the least deviation from control values. Cell viability, intracellular ATP levels, GAG release and NO production in human osteoarthritic chondrocytes were all comparable to control values when cultured in this O_2 tension over 96 hours. The work of Schneider et al (2004) similarly demonstrates the positive role that physiological oxygen tension exerts on chondrocyte survival. Culture of chondrocytes in low (1%) or high (21%) oxygen tensions led to significant cellular apoptosis, whilst culture in 5% O_2 was protective, maintaining cell number and limiting apoptosis over the culture period. In this study, lowering or raising O_2 levels to 2.5% or 21% led to non-significant reductions in cell viability although it has to be noted that our experimental culture duration was much shorter than that used by Schneider et al. Importantly however, reductions in O_2 tension to <1%, significantly reduced cell viability by 24 hours, highlighting the effect that removal of O_2 has on chondrocyte survival. Interestingly, the pH of media was also significantly reduced in anoxic conditions, an effect that was specifically related to the presence of chondrocytes, as cell-free conditions did not show any alteration to media pH over

96 hours. As extracellular pH closely correlates with intracellular pH and intracellular acidification has recently been shown to induce apoptosis in chondrocytes (Hu et al, 2012), the potential interactions between anoxia and extra/intracellular acidification on cell survival in OA chondrocytes requires further investigation.

Moreover, the addition of 10ng/ml IL-1β reduced cell viability, accentuating responses seen in IL-1β-free conditions. The pro-apoptotic effects of IL-1β on chondrocytes are well documented (Blanco et al, 1995; Yasahura et al, 2005; Zhou et al, 2008) and we demonstrate that this pro-apoptotic effect is oxygen sensitive as co-culture in IL-1β and hyperoxia or anoxia stimulated large reductions in cell viability, significantly different than those seen at 5% O_2, especially at 48 hours (table **3.3**, p96).

Glycosaminoglycan (GAG) release from human OA chondrocytes was also observed as being O_2 sensitive in this study. The culture of chondrocytes in anoxic, hypoxic and hyperoxic conditions led to significant increases in GAG release after 96 hours, an effect accentuated with addition of IL-1β. Culture of chondrocytes in 5% O_2 tension did not alter GAG release profiles over the time course studied but were significantly increased with IL-1β stimulation, an effect that was significant at 48 hours (see table **3.3**, p96). Of specific note was the finding that GAG release was significantly increased from control values in hyperoxic (21%) O_2 tensions and adds strength to the view that culture of chondrocytes in hyperphysiological O_2 tensions may be unsuitable for normal chondrocyte function (Henderson et al, 2010). In agreement, the work of Saini et al (2004) demonstrates that the three dimensional *in vitro* culture of chondrocytes at 5% O_2 significantly enhances GAG production and retention rate compared to values found at 21% O_2 in bovine chondrocytes (Saini et al, 2004). Likewise, expansion of rabbit chondrocytes in 5% O_2 has been shown to increase several ECM components compared to higher oxygen tensions after 3 weeks in culture (Henderson et al, 2010) and adds weight to the claim that ambient oxygen tensions are deleterious to chondrocyte ECM synthesis, with reductions to physiological oxygen tensions being beneficial.

It was observed that a significant reduction in intracellular ATP levels occurred when OA chondrocytes were exposed to hypoxic or anoxic conditions. This finding is implicit with reduced glycolysis under low oxygen tensions and demonstrates additional evidence for a 'negative Pasteur effect' in chondrocytes whereby reductions in oxygen tension from physiological levels causes significant inhibition of glycolysis and lactate production (Lee & Urban, 1997). The work of Lee and Urban (1997) also highlights the deleterious effects of hypoxia on proteoglycan synthesis, attributed to anoxia-induced ATP inhibition and relates well to findings from the current study that hypoxia and anoxia lead to significant increases in GAG release. Interestingly, oxygen levels of media cultured in 2.5% and 5% O_2 environments were unchanged after 96 hours, whereas we observed a reduction in oxygen tension to ~12% after 96 hours in 21% O_2 culture. This finding, twinned with the observation that hyperoxia led to non-significant increases in intracellular ATP levels may reflect alterations in chondrocyte metabolism, such as increased oxygen consumption or a metabolic switch towards upregulated oxidative phosphorylation, when exposed to an oxygen-rich environment (Benel et al, 1986; Marcus et al, 1973; Mignotte et al, 1991). This finding is particularly important when considering the role of appropriate oxygen tension in expansion and culture of cells primed for chondrogenesis and is fast becoming an area of intense research in tissue engineering aimed at therapeutics in OA (Dos Santos et al, 2010; Duval et al 2012; Grayson et al, 2007; Kanichai et al 2008).

Redox imbalance is a key feature in the aetiology and progression of joint diseases. As such nitrite and nitrate were used as markers for nitric oxide (NO) production. Systemically, NO is a stable free radical gas that acts as a diverse messenger molecule, regulating many biological functions. The balance between NO overproduction and decreased bioavailability is implicated in many disease processes. Increased levels of nitrite in the synovial fluid of OA and RA patients as well as upregulated expression of inducible nitric oxide synthase in the inflamed OA synovium have recently been identified (Bryan et al, 2009; Stuehr, 2004) and NO production is linked to chondrocyte apoptosis (Blanco et al, 1995; Notoya et al, 2000) and inhibition of ECM synthesis (Studer et al, 1999). In this study it was observed that a significant increase in nitrite release into culture media occurred

under hyperoxic conditions at 48 hours, indicative of augmented NO production. Conversely, anoxic culture led to significant reductions in NO production in all conditions studied across the time course. Endogenous NO is produced by the complex conversion of the amino acid L-arginine to citrulline being catalysed by the nitric oxide synthase enzyme. This process consumes O_2 as an electron acceptor, and thus, the presence of O_2 is a pre-requisite for NO production via this pathway and goes some way to explaining both the hyperoxic-induced increase in nitrite release at 48 hours and the inhibitory effect of anoxia in our study. The role of NO in cell death is somewhat equivocal with the data suggesting it is a key driver of apoptosis and others showing it to be protective in articular chondrocytes (Del-Carlo & Loeser, 2002). Anoxia significantly inhibited NO production in our study (even in the presence of IL-1β) and therefore it appears that NO-induced apoptosis alone cannot explain the marked cell death observed at this oxygen tension, and may indicate that the dual role of NO is likely to be oxygen-dependent.

The effect of extracellular pH on OA chondrocyte function

The effects of extracellular pH on chondrocyte function have received less attention than that of O_2 tension despite being a crucial modulator of cell function in other tissues (Arnett, 2010; Caso & Garlick, 2005; Terminella et al, 2002). Regular mechanical compression alters the extracellular ionic composition of cartilage, including local H^+ levels, which modulate chondrocyte membrane receptors and ion channel activity, ultimately modulating the mechanical properties of cartilage (Dascalu et al, 1993; Lui et al, 2002). Das et al (2010) showed that aggrecan, SOX9 and lactate production showed pH-dependency in human OA chondrocytes leading to the conclusion that extracellular pH had a stronger effect than oxygen tension on chondrocyte function, although it is to be noted that 5% O_2 was the lowest oxygen tension evaluated in this study which may explain some of their findings (i.e. a lack of HIF-1α expression). In other studies, cartilage matrix synthesis displays significant pH-dependency (Wilkins and Hall, 1995, Wu et al, 2007) and acidosis is linked to cellular apoptosis in chondrocytes (Yuan et al, 2010) and other cells (Furlong et al, 1997; Gottlieb et al, 1996). In early OA, the fixed charge density of cartilage is altered as proteoglycans are lost from the matrix, significantly altering

the ionic composition of cartilage (Mobasheri et al, 1998). ECM and synovial fluid acidosis are clinical features of OA that correlate with disease grade (Geborek et al, 1989, Levick, 1990, Mansson et al, 1990) and suggest pH should have an important role in maintaining cartilage integrity. In the present study, acidosis was an important regulator of OA chondrocyte cell viability, GAG release, intracellular ATP production and NO production.

It was found that incubation of HOAC in extracellular acidosis significantly reduced chondrocyte viability after 96 hours. Culture in acidosis and hyperoxia lead to significant reductions in cell viability at 96 hours which is in accordance with the recent work of Rong et al (2012) who demonstrated significantly increased chondrocyte apoptosis and expression of pro-apoptotic genes in rat chondrocytes cultured in acidosis and ambient oxygen conditions (for 3 hours) (Rong et al, 2012). These observations were extended by demonstrating exacerbated cell death in acidosis and hypoxia/anoxia. Incubation in physiological O_2 tensions (5%) maintained cell viability in the present study even in the presence of an acidic challenge, highlighting the potentially protective effect that culture at physiological O_2 levels can have on OA chondrocytes. Significant increases in GAG release also occurred in acidosis after 96 hours, accentuated by the addition of IL-1β. This effect was particularly noticeable in physiological oxygen tensions where GAG release profiles were increased >2 fold compared to acid-free conditions. These findings are in accordance with those of other authors who have demonstrated the inhibitory effect of extracellular acidosis on GAG synthesis and ECM gene expression (Das et al, 2008; Wilkins and Hall, 1995; Wu et al, 2007).

Interestingly, intracellular ATP levels appeared pH sensitive, particularly in higher oxygen tensions. Intracellular ATP levels were significantly reduced from control in 5% and 21% O_2 conditions (being significantly lower than values in acid-free conditions). Inhibition of glycolysis by low pH has been observed in other tissues (Bevington et al, 1995) which supports the present finding and suggests reduced energy synthesis in low pH. In addition, increased GAG release was observed in these conditions and therefore provides potential evidence for an acidosis-mediated inhibition of intracellular ATP levels in an oxygen sensitive manner, which may

account for the altered GAG profiles observed. Alternatively, increased NO production in higher oxygen tensions may partially explain the observed reduction in ATP_i as NO has been shown to inhibit GAPDH (Stefanovic-Racic et al, 1994), a key enzyme in the formation of ATP by glycolysis. Thus the results may point to an acidosis induced inhibition of intracellular ATP levels through stimulation of NO, which is oxygen-dependent. Clearly the role of extracellular pH and oxygen tension on key functions such as cell survival, energy metabolism and ECM homeostasis are complex, but appear to be interactive. More work is needed to fully elucidate the extent of these interactions in normal and diseased cartilage and may lead to a better understanding of the relationships between oxygen, pH, NO, energy production, cell survival and matrix synthesis in cartilage.

3.6 Conclusion

To conclude this part of the study, a three dimensional *in vitro* chondrocyte model was developed to study the effects of oxygen and pH on cell viability and redox balance in human osteoarthritic chondrocytes. This work demonstrates the concurrent effects of O_2 tension and pH on cell viability, ATP_i, GAG release and NO production in HOAC. An O_2 tension of 5% provided the most suitable environment for chondrocyte homeostasis. When O_2 tension was lowered or raised from this parameter cell viability was reduced and GAG release was increased. Culture in acidosis exacerbated these responses, especially in low oxygen environments. NO production displayed oxygen sensitivity, being inhibited in very low oxygen tensions and augmented as oxygen levels were increased. Finally, chondrocytes appear to increase their O_2 consumption when the concentration of O_2 becomes abundant, which may indicate altered metabolic function, but also eventually resulted in increased GAG release and reduced cell viability. This work demonstrates the importance of studying both O_2 tension and pH conditions on chondrocyte cell function. The mechanisms behind the observed O_2 and pH-sensitivity require further work.

Chapter 4

The effect of antioxidants to alter oxygen and pH-sensitivity of human osteoarthritic chondrocytes in a 3-D alginate bead culture system

Aspects of this chapter have been published (see **appendix ii** for full article):

Collins, JA., Moots, RJ., Winstanley, R., Clegg, PD., Milner, PI., (2013). Oxygen and pH-sensitivity of human osteoarthritic chondrocytes in 3-D alginate bead culture system. *Osteoarthritis and Cartilage* [ahead of print] DOI: 10.1016/j.joca.2013.06.028

4.1. Introduction.

Mitochondria play an essential role in many cellular processes including ATP synthesis through oxidative phosphorylation, calcium homeostasis and apoptosis. Due to the hypoxic nature of cartilage however, oxidative phosphorylation is low in chondrocytes which is reflected by a low mitochondrial density and a low O_2 consumption rate (Mignotte et al, 1991). Despite this, chondrocytes require and utilise O_2, and the mitochondrial electron transport chain (ETC) is active although it may not express a full complement of ETC protein complexes (Mignotte et al, 1991). With the reduced contribution of oxidative phosphorylation in cellular energy production in articular chondrocytes, the importance of mitochondria in other roles such as calcium regulation and cell survival are being increasingly recognised (Terkeltaub et al 2002).

Crucially, mitochondria are the main site of ROS generation in cartilage (Pieczenik & Neustadt, 2007). Mitochondria respond to changes in substrate availability and therefore display oxygen-sensitivity. Such changes alter the electron flux through the ETC and modulate mitochondrial polarisation and ROS production (Blokhina et al, 2010; Milner et al, 2007) and provide a role for oxygen in this hypoxic environment. Coupled electron transfer and proton extrusion is fundamental for mitochondrial homeostasis. Electron transfer between ETC complexes however is not totally efficient and electrons may be 'leaked' out of the ETC, resulting in the transfer of electrons to oxygen, forming ROS. Despite the well-documented deleterious effects of ROS overproduction and concomitant oxidative stress, recent evidence demonstrates that sustained and controlled levels of ROS are required for cellular homeostasis. In isolated articular chondrocytes, short term (3hr) hypoxic culture has been shown to inhibit ROS generation, leading to intracellular acidosis (Milner et al 2006). As such, O_2-dependent ROS production is beginning to be viewed as a key intracellular signal capable of modulating cellular function e.g. through interactions with HIF's (Schipani et al, 2001) or NRF-2 release (Stepkowski & Kruszewski, 2011) and may be altered in many pathologies where hypoxia is a known feature (Li et al, 2012).

The role of extracellular acidosis on ROS-sensitive pathways in cartilage has received little attention. ROS generation shows pH dependency in a variety of cell types (Pekun et al, 2012; Reimann et al, 2011; Zablocki et al, 2005) and extracellular acidosis has the ability to inhibit antioxidant enzyme activity (Ying et al, 1999). In OA, joint acidosis is a recognised event, the degree of which correlates with disease grade (Levick et al, 1990). In addition, results in Chapter 3 suggest that acidosis modulates human OA chondrocyte ATP_i levels, survival and RNS production (NO). Taken together, it is hypothesised that this environmental parameter may exert control over redox sensitive pathways and mitochondrial function in chondrocytes and requires identification. Revealing the distinct and interactive relationship between varying oxygen tensions and extracellular pH on OA chondrocyte mitochondrial function and redox balance remains unexplored, but may increase our understanding of the pathophysiology of joint disease.

Ordinarily, antioxidant enzymes (SOD, GPx) present within cartilage are well equipped to detoxify ROS and maintain redox equilibrium (Afonso et al, 2007; Gao et al, 2008). Changes in the expression of SOD, glutathione and GPx are seen in ageing and OA tissues (Del-Carlo & Loeser, 2003; Scott et al, 2010) and appear sensitive to fluctuations in oxygen and pH in other cell types (Davis et al, 1989; Mapp et al, 1995; Pekun et al, 2012; Tsunoda et al, 2013; Ying et al, 1999). In an attempt to replenish antioxidant enzymes and counter disease-associated oxidative stress, the addition of exogenous compounds that target redox pathways provides a promising avenue of research for disease modification. In chondrocytes, exogenous antioxidant addition to maintain redox balance has received broad attention, with variable degrees of success (Atkuri et al, 2007). The role of N-acetylcysteine and resveratrol as redox regulators appear particularly intriguing due to their diverse reported antioxidant properties (Dave et al, 2008; Nakagawa et al, 2009). Both compounds appear to modulate different ROS-sensitive pathways in chondrocytes and therefore represent potential candidates as therapeutic agents for OA (Dave et al, 2008; Nakagawa et al, 2009).

The sensitivity of mitochondrial function to alterations in oxygen and pH in other cells (e.g. Pekun et al, 2012; Zablocki et al 2005) and their links to cellular function

through regulation of ROS levels and redox balance make them an attractive organelle to examine in relation to chondrocyte function and the pathogenesis of OA (Blanco et al 2011). Accordingly, this chapter examines the effects of O_2 tension and pH on cellular redox status and mitochondrial function in OA articular chondrocytes using the *in vitro* model developed in chapter 3. Additionally, the ability of exogenous antioxidant addition (resveratrol and N-acetylcysteine) to alter redox status and mitochondrial function were analysed.

4.2. Aims

The aims of this part of the project were:

(1) To study the effect of varying O_2 tensions, pH and IL-1β on mitochondrial function and redox balance in HOACs by measuring the mitochondrial membrane potential, ROS generation and the GSH:GSSG ratio of HOAC after 48 hours. Chondrocyte homeostasis (intracellular pH, glycosaminoglycan release into media and cell viability) and phenotype were also assessed (COL2A1, SOX9 and COMP protein expression) before and after 48 hour experimental culture.

(2) To analyse the ability of exogenous antioxidant (AOX) addition (resveratrol and N-acetylcysteine) to alter mitochondrial function and redox status in HOACs exposed to varying oxygen tensions, pH and IL-1β stimulation for 48 hours.

(3) To quantify redox-specific and hypoxia-sensitive proteins in HOACs (namely, SOD1, SOD2, KEAP-1, NRF-2, HIF-1α) and to assess changes in expression as a result of altered oxygen tensions, pH and IL-1β stimulation.

4.3. Study Design

- **Cell culture and experimental culture conditions**

Based on the results obtained over the 96 hour incubation period (chapter 3), 48 hours represented a consistent time point for alterations in chondrocyte function in response to hypoxia and acidosis. Therefore all further measurements were performed after 48 hours incubation only. Human osteoarthritic articular cartilage was obtained from knee joints removed by total-knee arthroplasty. Tissue was obtained from a total of 15 donors (7 male aged 62-85 and 8 female aged 55-86, mean age 70.6 ± 2.5). Since monolayer expansion of cell numbers were not necessary, primary chondrocytes were used. Primary HOAC were freshly isolated and cell viability was determined by Trypan blue exclusion and was >95% for all donors. Primary cells were cultured in 3-D alginate beads (as previously described (section **2.2.5**)) and maintained in a 5% O_2 tension for 14 days. Alginate beads were then incubated in pH 7.2 (normal media) or pH 6.2 (acidosis) conditions in the absence or presence of IL-1β and in the absence or presence of NAC (2mM) or resveratrol (10μM) for 48 hours at <1%, 2%, 5% or 21% O_2 conditions. Following experimental incubation, O_2 and extracellular pH measurements of media were taken and the remaining media sample frozen (-20°C) for analysis of GAG and LDH release. Alginate beads were released from their alginate matrix by depolymerisation in sodium citrate solution and immediately analysed.

- **Western blotting**

The chondrocyte phenotype was characterised by analysing COL2A1, COMP and SOX9 protein expression. Primary antibodies against SOD1, SOD2, HIF-1α, KEAP-1 and NRF2 were also used for detection of redox specific and hypoxia regulated proteins. α-tubulin was used as a loading control (see materials and methods for antibody details (table **2.1**)).

- **Cell media analysis**

The DMMB assay was used to assess the concentration of sulphated GAG in media samples. Cell viability was quantified using the Cytotoxicity Detection Kit ^{Plus} as per the manufacturer's instructions.

- **Measurement of reactive oxygen species (ROS) generation**

Intracellular ROS were measured using the Dichlorofluorescin (DCF) method. Chondrocytes were pelleted, washed and seeded at $1x10^6$ in pre-equilibrated sterile saline solution then incubated with DCFH-DA and fluorescence measured (excitation at 490nm/emission 535nm).

- **Determination of the mitochondrial membrane potential (ΔΨm)**

ΔΨm was assessed using JC-1. Chondrocytes were mixed with JC-1, for 40 minutes and then entered onto individual cover slides. Specific filters for rhodamine and flourescein were used to determine mitochondrial polarisation.

- **Measurement of the GSH:GSSG ratio**

The GSH:GSSG ratio was determined by the GSH:GSSG-Glo™ Assay following the manufacturer's instructions. Average RLU values for GSH and GSSG were used to determine the ratio of GSH to GSSG in unknown samples.

- **Intracellular pH measurement**

The BCECF method was used to determine intracellular pH of HOAC. Chondrocytes were incubated with BCECF-AM for 30 minutes and fluorescence was spectrophotometrically measured (excitation at 490nm, emission at 535nm).

- **Statistical analysis**

Significant differences were determined by One-way ANOVA with a Dunnett's and Tukey HSD *post-hoc* correction. A minimal significance level of $P =< 0.05$ was used. Unless otherwise stated, results are presented as % compared to control (5% - O_2, pH7.2, time = 0).

4.4. Results

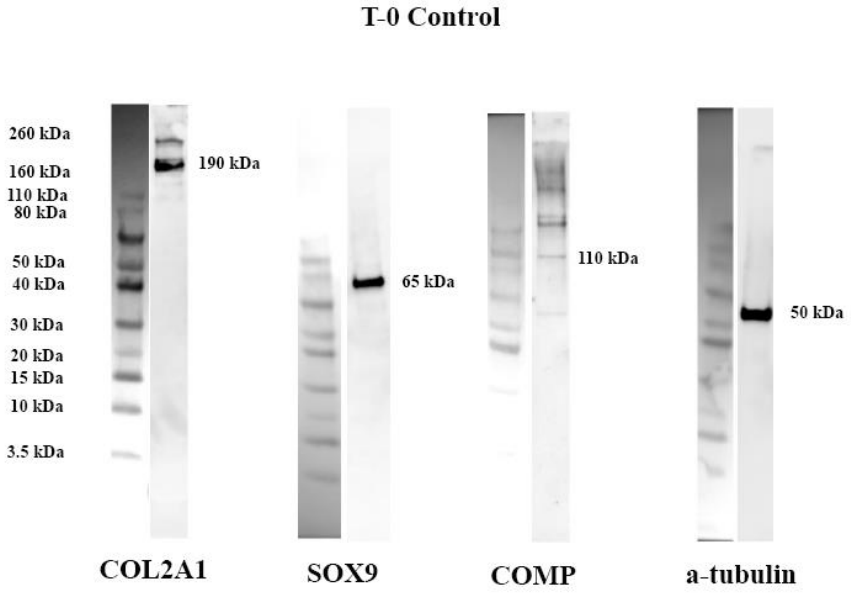
4.4.1. Analysis of cell media and chondrocyte phenotype

To assess the ability of primary cultured osteoarthritic chondrocytes to express markers of a chondrocyte phenotype after 14 days in alginate bead culture, a panel of chondrocyte phenotype markers were used (COL2A1, SOX9 and COMP protein expression) at time-zero and after 48 experimental incubation, in either pH 7.2 or 6.2 conditions. The oxygen consumption of HOAC after experimental incubations under varying oxygen tensions and pH conditions in the absence or presence of IL-1β or the antioxidant (AOX) compounds N-acetyl cysteine or resveratrol (48 hours) was also measured. GAG and LDH release (marker of cell viability) into media were also determined after 48 hours.

Chondrocyte phenotype

Primary HOAC lysates (T=0) after 14 days in alginate culture continued to express markers of an articular chondrocyte phenotype. Figure **4.1A** shows representative expression of COL2A1, SOX9 and COMP protein expression from one donor indicating expression of ECM components over the 14 day culture period in alginate beads. Figure **4.1B** demonstrates COL2A1, SOX9 and COMP protein expression after 48 hours experimental incubation in either normal (pH7.2) or acidic (pH 6.2) conditions and shows homogeneity in protein expression between donors. Under pH 7.2 conditions, band densitometry (figure **4.1C**) shows no significant differences in COL2A1, SOX9 or COMP protein expression after 48 hours culture in <1%, 2%, 5% or 21% O_2 conditions (compared to control). When cultured in acidosis (pH 6.2), COL2A1 protein expression was significantly reduced compared to control in oxygen tensions below 21% O_2 (all P=<0.05). SOX9 protein expression was also significantly reduced in acidosis after 48 hours in anoxic culture (P=0.008) (figure **4.1D**).

A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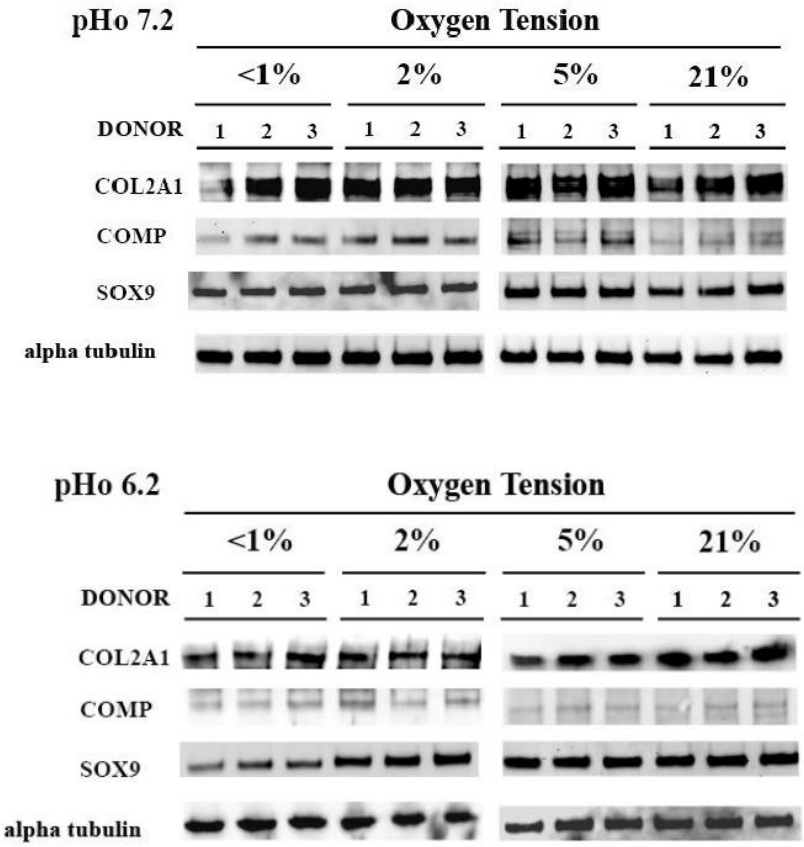
SOX9
COMP
a-tubulin

B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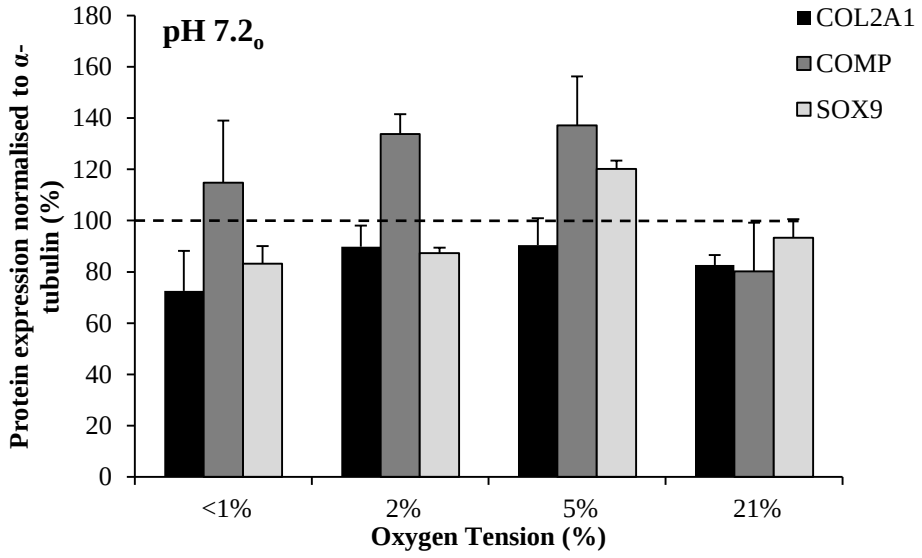


D

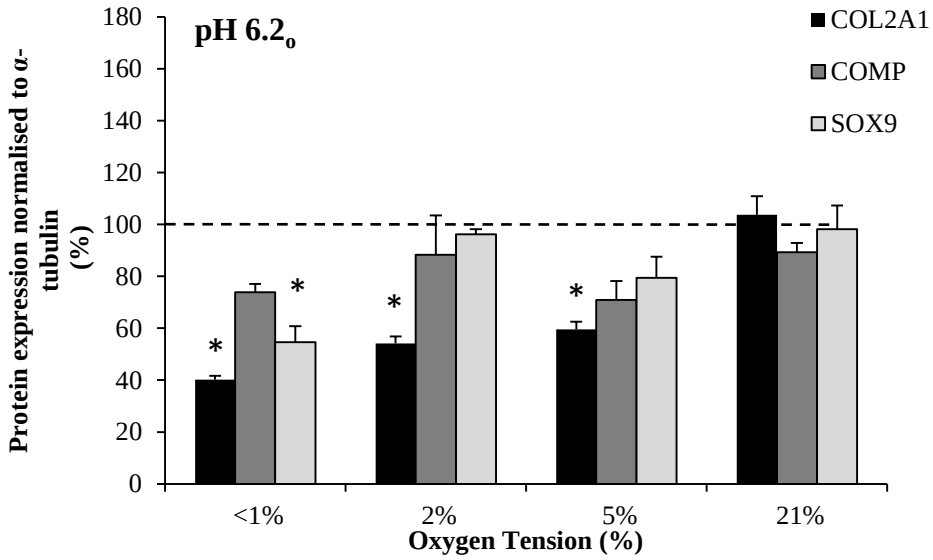


Figure 4.1. Chondrocyte phenotype of primary HOAC cultured in three-dimensional alginate beads. Western blotting for COL2A1, SOX9 and COMP was performed on control cell lysates taken on day 14 of the alginate culture period (t=0) (**A**) (representative full lane blot). HOAC were then incubated in <1%, 2%, 5% or 21% O_2 in normal (pH 7.2) or acidic (pH6.2) media for 48 hours (n=3) and expression of COL2A1, SOX9 and COMP was identified (**B**). Band densitometry was used to quantify protein expression in cells cultured in pH 7.2 (**C**) and pH 6.2 (**D**) conditions. Data was normalised to α-tubulin and presented as percentage compared to control (T=0). *P=<0.05 compared to control (time-0).

Oxygen concentration of media

The concentration of O_2 in media samples and is shown in Table **4.1**. No effect of pH or IL-1β was found on O_2 levels in AOX untreated or AOX treated conditions. Consequently, data were pooled. The presence of the antioxidants N-acetylcysteine or resveratrol did not alter media oxygen concentrations following 48 hour incubation.

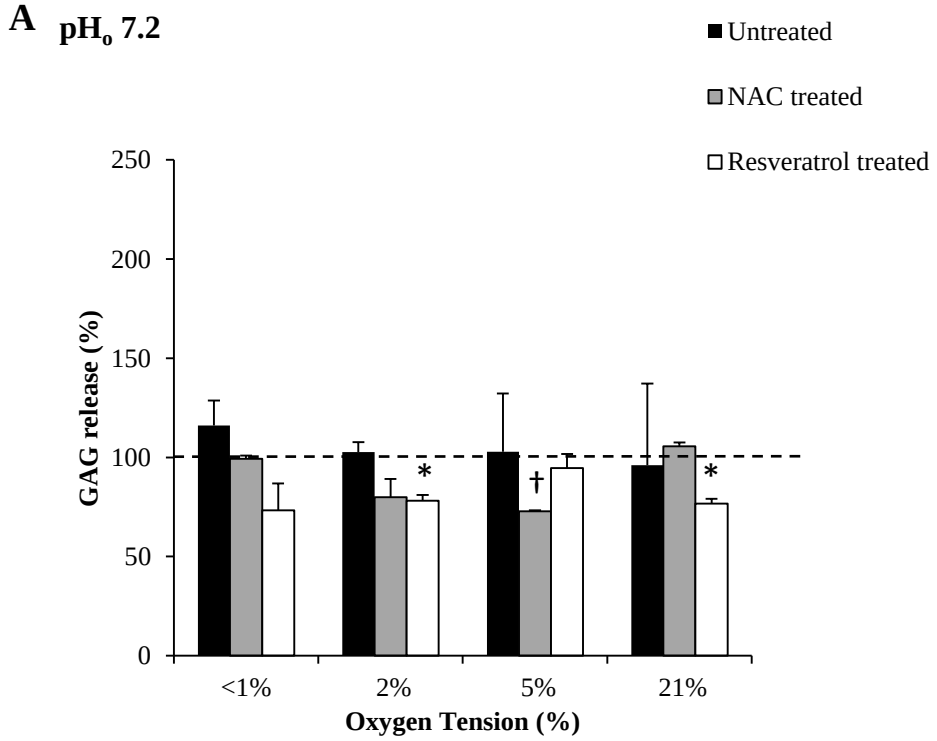
Mean O_2 tension of spent media				
	<1%	**2%**	**5%**	**21%**
Untreated	0.54 ± 0.22	1.86 ± 0.33	5.00 ± 0.26	14.49 ± 0.17
NAC treated	0.58 ± 0.34	1.71 ± 0.51	6.14 ± 1.02	15.02 ± 0.26
RES treated	0.67 ± 0.28	1.64 ± 0.29	6.36 ± 0.07	12.79 ± 0.04

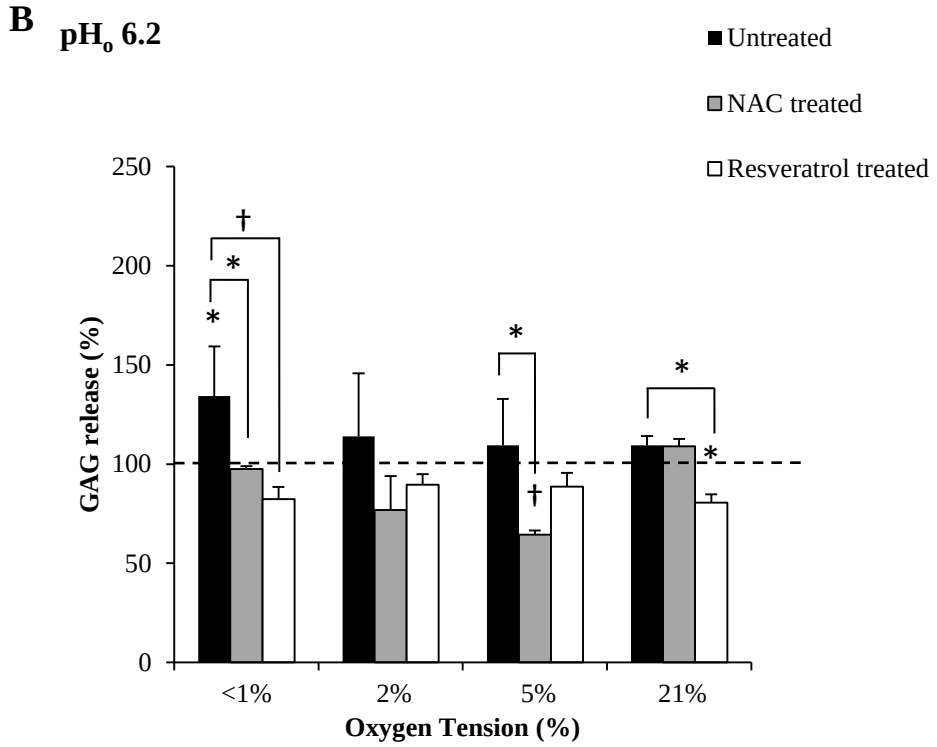
Table 4.1. O_2 levels of media after experimental incubations. Primary HOAC were cultured in alginate beads for 14 days and then incubated in <1%, 2%, 5% or 21% O_2 in normal (pH 7.2) or acidic (pH6.2) media in the absence or presence of 10ng/ml IL-1β, resveratrol (10µM) or N-acetylcysteine (2mM) for 48 hours. Aliquots of cell culture media were harvested immediately after experimental exposure and O_2 levels were estimated using a Hansatech oxygen electrode (following calibration). Results represent mean ± SEM O_2 levels after 48 hours (n=3 donors).

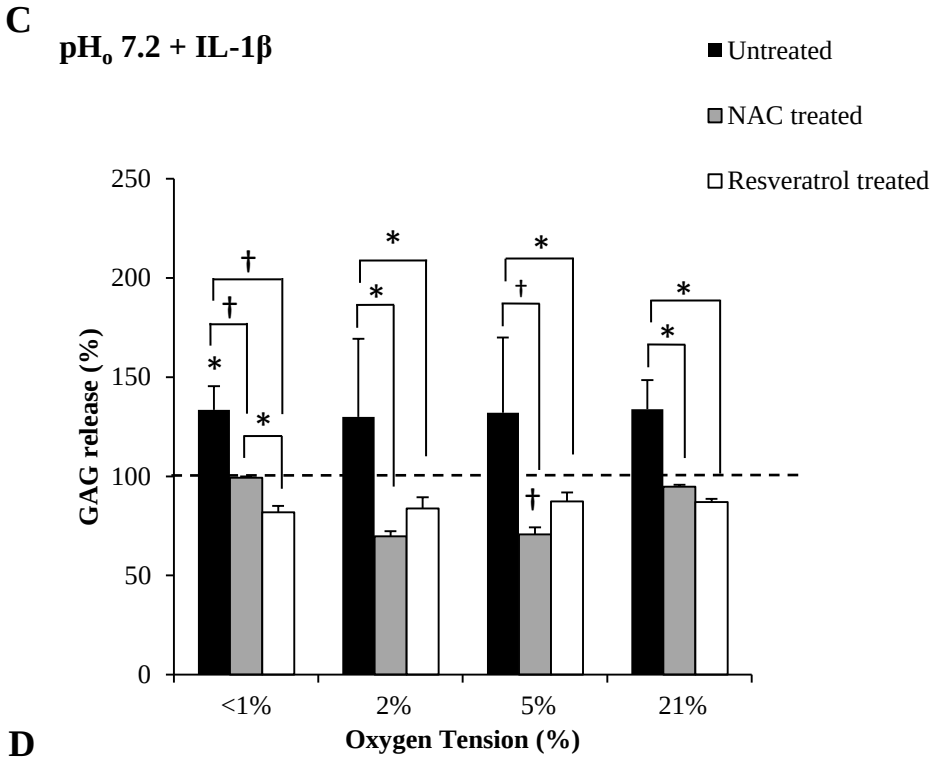
Glycosaminoglycan release

GAG release was measured as a marker of proteoglycan degradation. The data demonstrate a significant effect of anoxia on GAG release under acidic conditions and the ability of resveratrol and NAC to reverse this increased GAG release under certain oxygen tensions. In AOX untreated conditions (*black bars*), GAG release was unchanged from control values in all oxygen tensions (in pH 7.2 media), however anoxic and acidic conditions led to significant increase in GAG release (figure **4.2A, B**). Interestingly, the addition of NAC (2mM) (*grey bars*) and resveratrol (10uM) (*white bars*) led to a reduction in GAG release at pH 7.2 in some oxygen conditions and prevented the rise in GAG release in acidic and anoxic conditions.

The addition of IL-1β caused a significant increase in GAG release in hypoxic (pH 6.2) and anoxic conditions (pH 7.2 and pH 6.2) (figure **4C** and **D**). In every condition studied, IL-1β stimulated an increase in GAG release in AOX untreated chondrocytes that was significantly reduced in the presence of N-acetyl cysteine and resveratrol (figure **4.2C** and **D**). The results of this part of the study show that the antioxidants N-acetyl cysteine and resveratrol are powerful mediators of GAG release, particularly in the presence of the pro-inflammatory cytokine, IL-1β, but they also modulate anoxic and acidic-mediated release of GAGs. Additionally, these antioxidants also appear to suppress basal GAG release, suggesting the importance of redox systems in GAG (synthesis and) release.


A
pHo 7.2
Untreated
NAC treated
Resveratrol treated
250
200
150
100
50
0
GAG release (%)
*
†
*
<1%
2%
5%
21%
Oxygen Tension (%)


B
pHo 6.2
Untreated
NAC treated
Resveratrol treated
250
200
150
100
50
0
GAG release (%)
†
*
*
*
*
*
†
<1%
2%
5%
21%
Oxygen Tension (%)



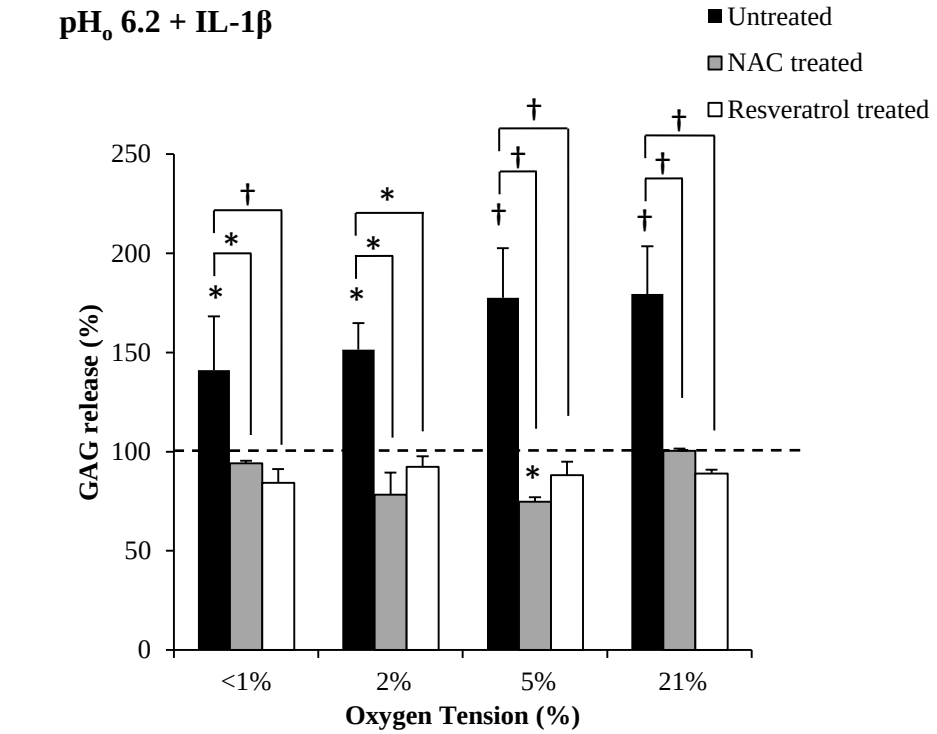


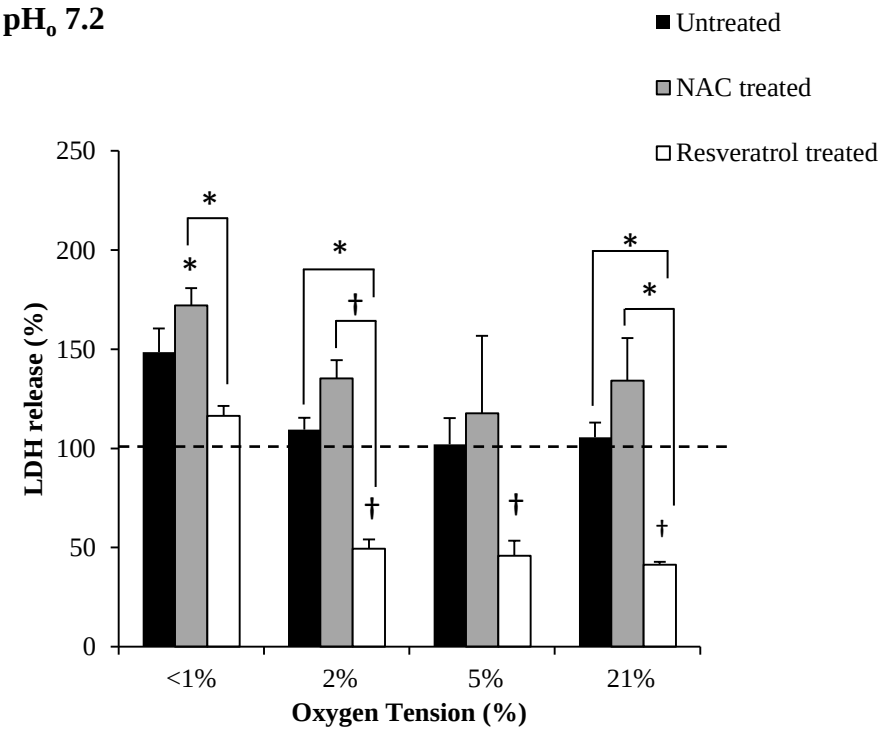
Figure 4.2. Effect of O_2 tension, pH, IL-1β stimulation and AOX compounds on HOAC GAG release after 48 hours. GAG release was determined by the DMMB method and presented as mean values compared to control (time=0, 5%O_2, pH7.2). HOAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and GAG release was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

Cell viability

Cell viability was determined by LDH release into media. No significant change in cell viability was measured at pH 7.2 or pH 6.2 under all oxygen conditions. The presence of IL-1β in acidic (pH 6.2) and anoxic (<1% O_2) conditions resulted in significant LDH release and hence reduction in cell viability (162.1 ± 11.5, *P*=0.023). In the presence of resveratrol, LDH release was markedly reduced but only at 2%, 5% and 21% O_2 where values were consistently reduced to <50% of control. Under anoxic conditions, resveratrol did not appear to exert any effect. N-acetyl cysteine had no effect on LDH release in 2%, 5% or 21% O_2 levels. Culture in anoxia however, enhanced LDH release, particularly in acidosis (with or without IL-1β) (209.6 ± 8.7% (*P*=0.006) and 204.5 ± 10.22 (*P*=0.001)).Thus it appears that resveratrol and N-acetylcysteine have different actions on LDH release and there is some oxygen-sensitivity to these compounds, particularly under very low oxygen conditions.

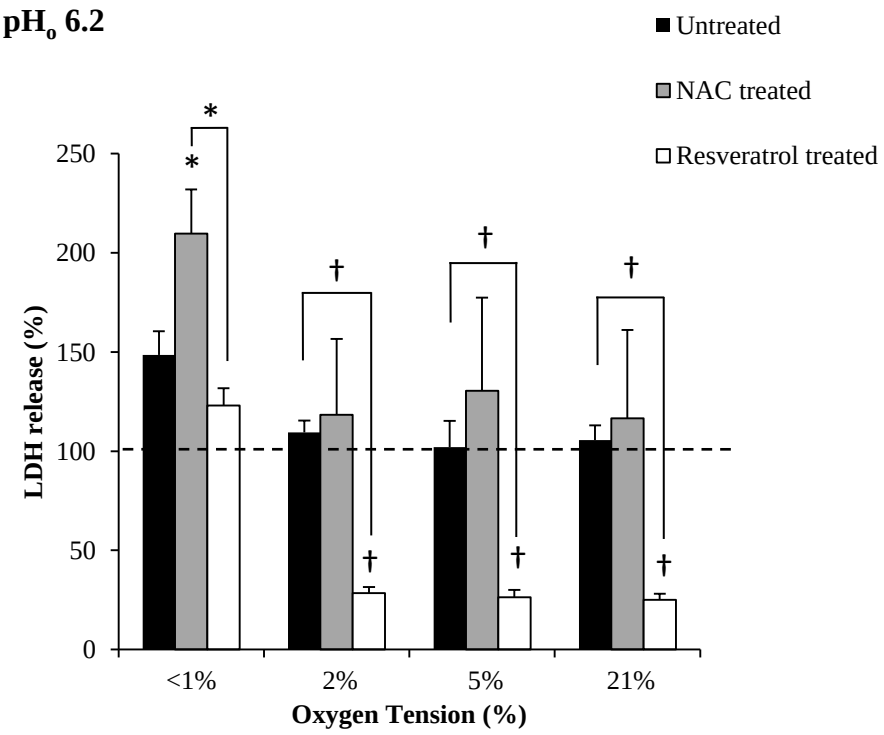
A

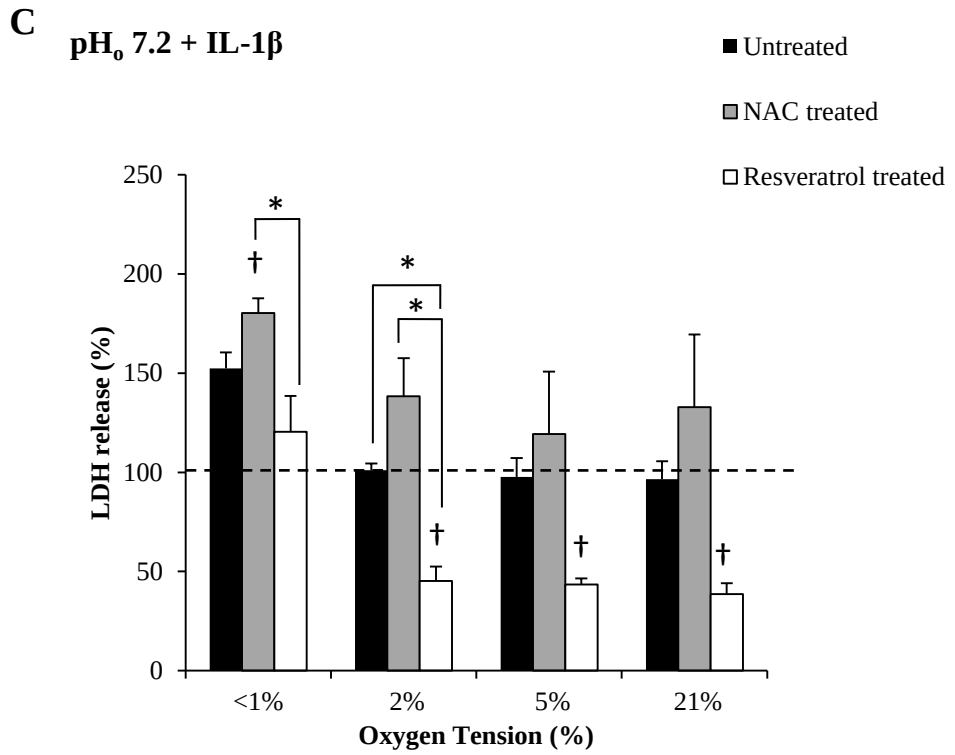
pH_o 7.2



B

pH_o 6.2





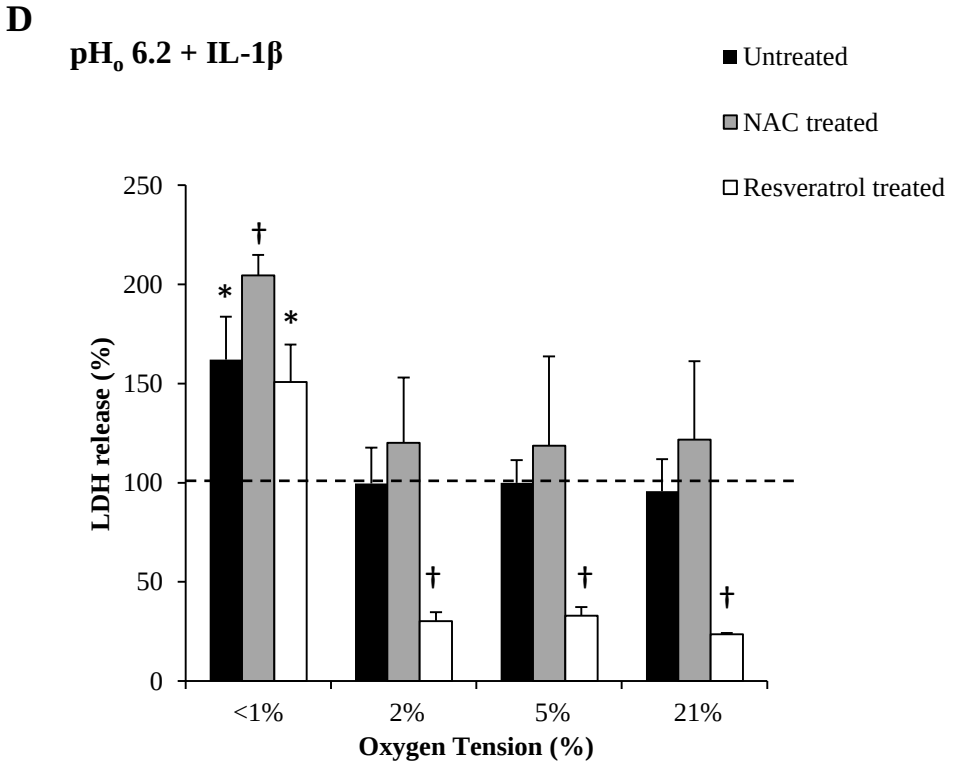


Figure 4.3. Effect of O_2 tension, pH, IL-1β and AOX compounds on HOAC LDH release after 48 hours. Cell viability was determined by LDH release and presented as mean values compared to control (time=0, 5%O_2, pH7.2). HOAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (**B**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and LDH release was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

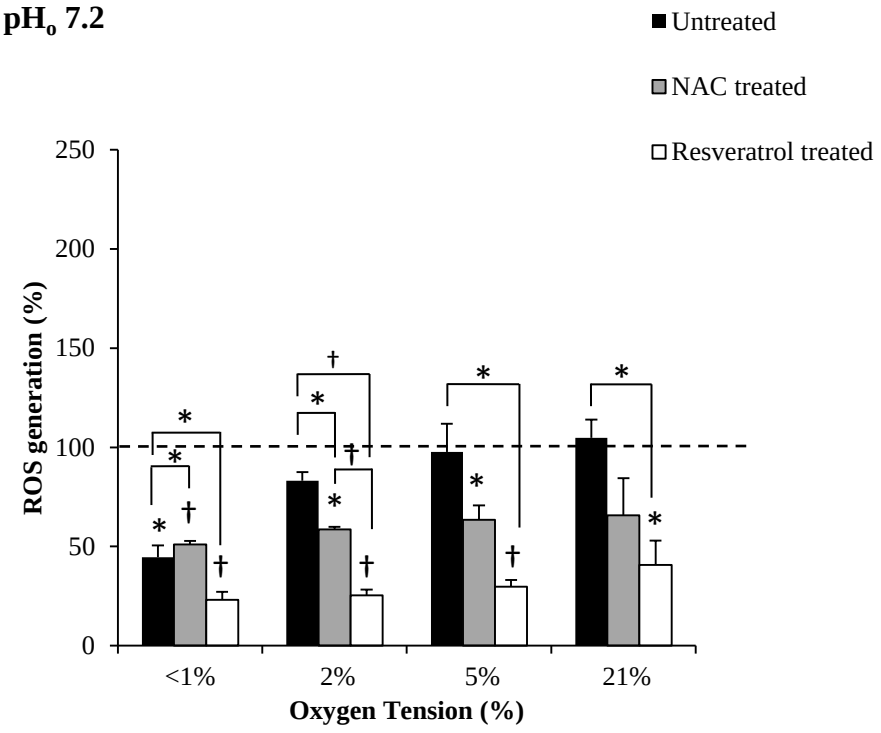
4.4.2. ROS generation

ROS generation is a key marker of the redox status of a tissue and despite being crucial in cell and tissue destruction in pathological states, may also participate in cell signalling and oxygen sensing (Milner et al, 2007). ROS production displays oxygen and pH sensitivity in cartilage and is implicated in the pathology of OA (Milner et al, 2007; Pekun et al, 2012; Reimann et al, 2011).

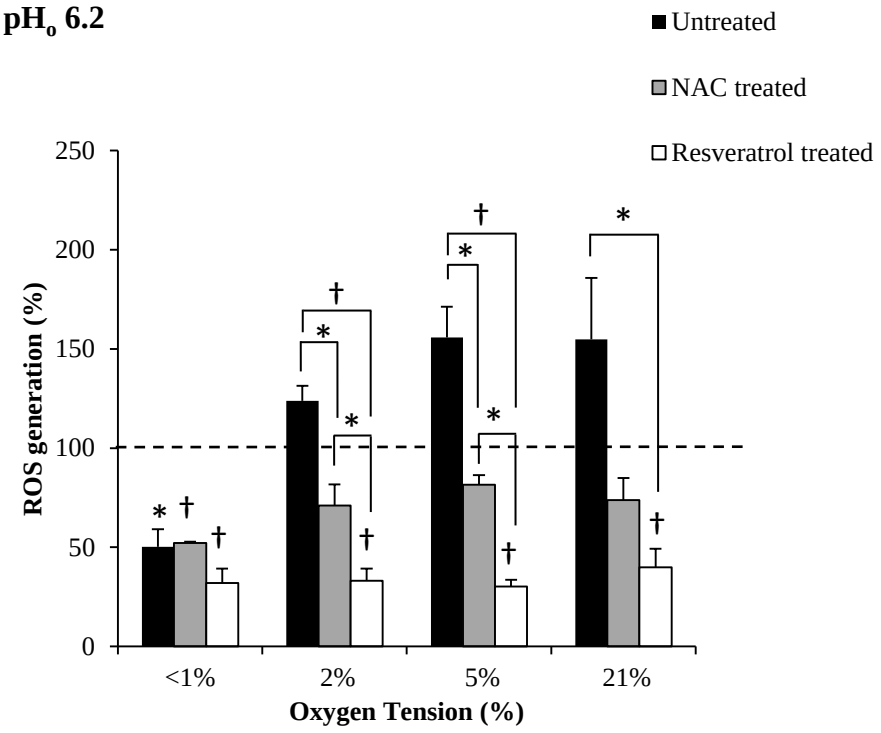
ROS generation at pH 7.2 in HOAC showed oxygen-sensitivity with reductions in ROS occurring with reducing oxygen levels, reaching statistical significance under anoxic conditions (Figure **4.4A**). Under acidic conditions however, ROS generation were increased (but only at 2%, 5% and 21% O_2), although these were not statistically significant (figure **4.4B**). In anoxic conditions, ROS levels were still suppressed, even in an acidic environment. Thus, although ROS levels show pH-sensitivity, there appears to be a stronger dependence on oxygen availability, which is consistent with previous reports (Milner et al, 2006, 2007). The presence of IL-1β enhanced ROS levels further, particularly in 5% and 21% oxygen conditions (figure **4.4.C** and **D**).

Treatment with NAC reduced ROS generation, particularly at 2%, 5% and 21% O_2. In anoxic conditions (<1% O_2), ROS levels were similar to untreated levels. NAC also reduced acid-induced and IL-1β induced increase in ROS generation, again at 2%, 5% and 21% O_2 levels. Addition of resveratrol markedly inhibited ROS generation to approximately 20-40% of control values. Again, this effect was most evident at 2%, 5% and 21% O_2. From these results, both N-acetyl cysteine and resveratrol (in particular) are strong suppressors of induced ROS release, even suppressing basal, non-stimulated ROS levels.

A **pH_o 7.2**



B **pH_o 6.2**



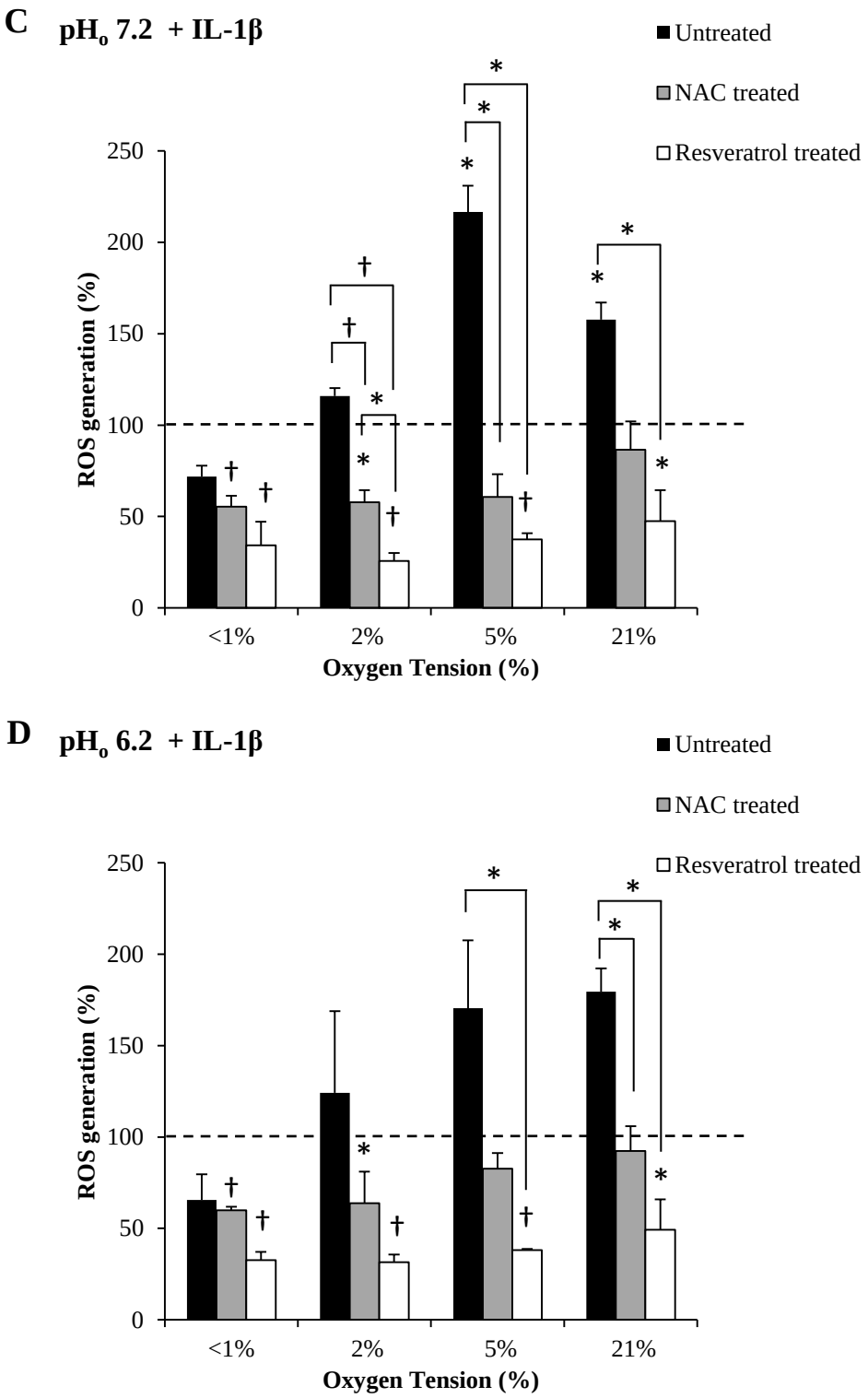


Figure 4.4. Effect of O_2, pH, IL-1β and antioxidant compounds on HOAC ROS generation. ROS generation was determined by the DCF method and presented as mean values compared to control (time=0, 5%O_2, pH7.2). HOAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and DCF fluorescence was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

4.4.3. Mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) is a key indicator of mitochondrial function. Since mitochondrial membrane polarisation is important in ROS generation, the following experiments investigated the effects of O_2 tension, pH, IL-1β and AOX compounds on ΔΨm in HOACs using the cationic dye JC-1 (see figure **4.5** for representative images).

Incubation at 5% or 21% O_2 (pH 7.2) maintained ΔΨm to control values after 48 hours incubation (92.6 ± 2.5% and 87.9 ± 3.2%, both ns). Reductions in oxygen tension led to significant reductions in red:green ratio indicating mitochondrial membrane depolarisation (figur**e 4.6A)**. At <1% and 2% O_2 tensions (pH 7.2), $\Delta\Psi_m$ was 32 ± 1.7% and 52 ± 3.6% of control, respectively (both P=<0.001). Acidosis significantly depolarised mitochondria in all AOX untreated conditions regardless of oxygen level (figure **4.6B**). Similarly, the presence of IL-1β also led to significant mitochondrial depolarisation (Figure **4.6 C** and **D**).

Addition of N-acetyl cysteine did not significantly alter mitochondrial membrane potential from untreated samples, under all conditions. Resveratrol, however, resulted in a reduction in the depolarisation of the mitochondrial membrane potential that occurred under hypoxic (2% O_2) conditions and in acidic conditions at 2%, 5% and 21% O_2. In the presence of IL-1β, resveratrol partially restored ΔΨm, particularly in anoxic and hypoxic conditions (figure **4C** and **D**).

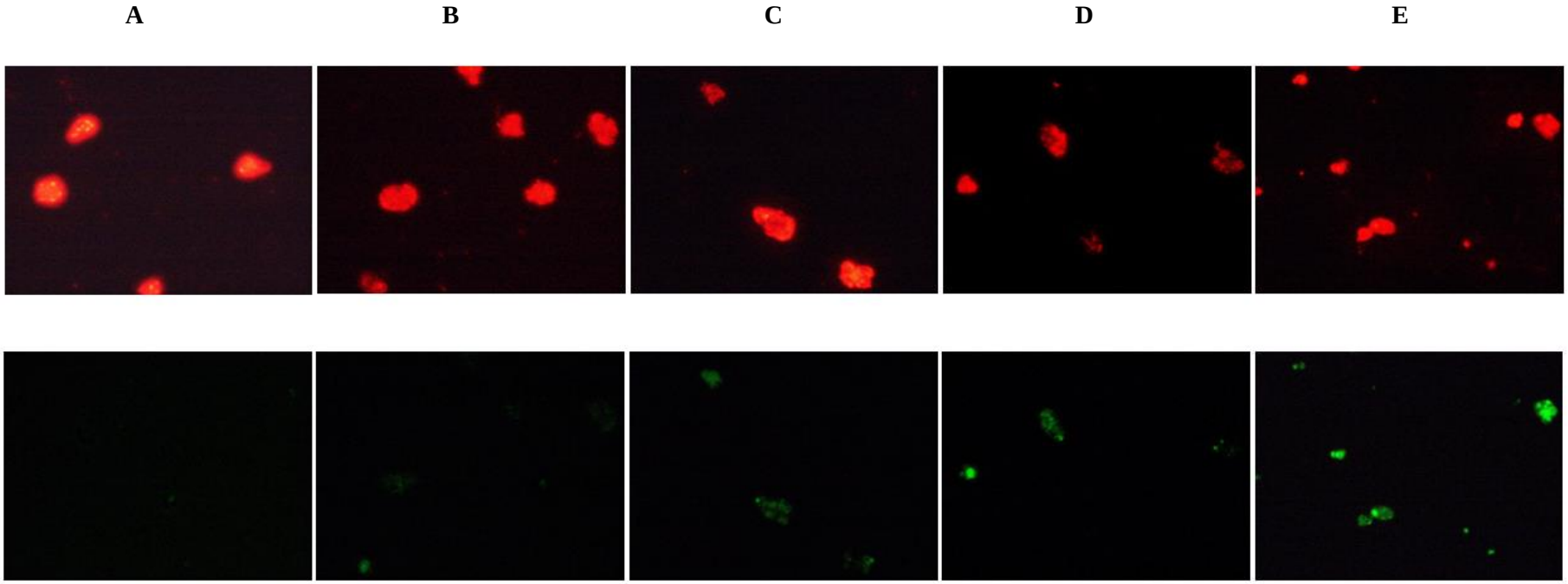
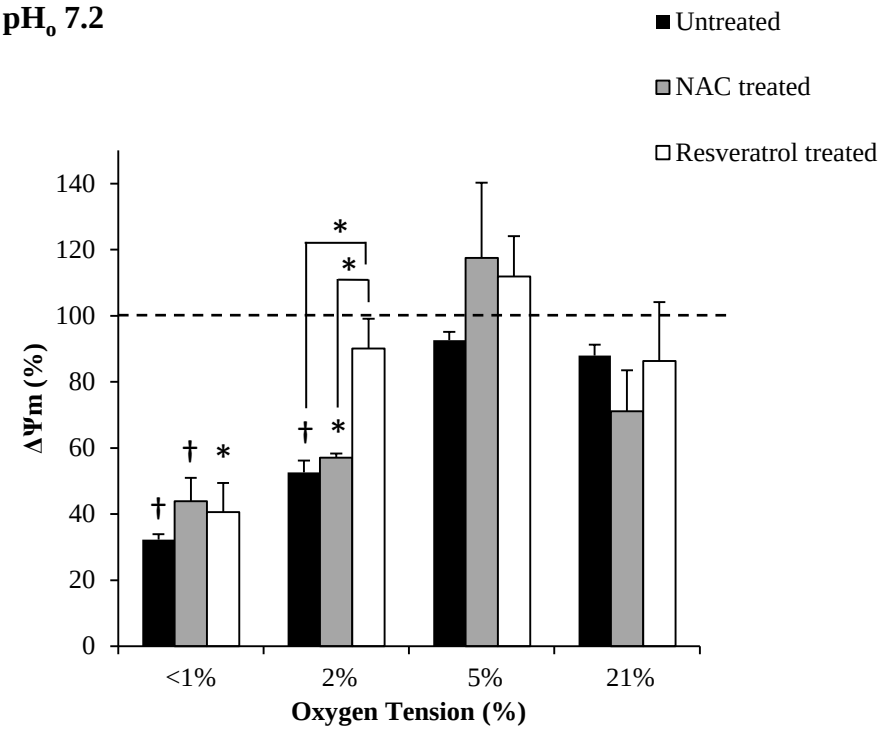


Figure 4.5. Representative images from 1 experiment of HOAC cultured in 5% O_2 and incubated with the fluorescent dye JC-1 to assess the ΔΨm. Panel **A** represents time-0 (control). Panels **B** and **C** represent cells cultured in pH 7.2 and pH 6.2 conditions. Panels **D** and **E** show cells cultured in pH 7.2 and pH 6.2 conditions in the presence of IL-1β (10ng). Upper panels (red fluorescence) demonstrate the formation of red JC-1 aggregates formed when the mitochondria is polarised. During mitochondrial depolarisation the JC-1 cannot accumulate and remains in its monomeric form in the cytoplasm and fluoresces green (Lower panels). The ratio of red: green pixel intensity was used to determine mitochondrial polarisation. Note the depolarisation (increase of green fluorescence) in the presence of IL-1β in both normal and acidic pH (panels **D** and **E**) and mild depolarisation in acidosis alone (panel **C)**.

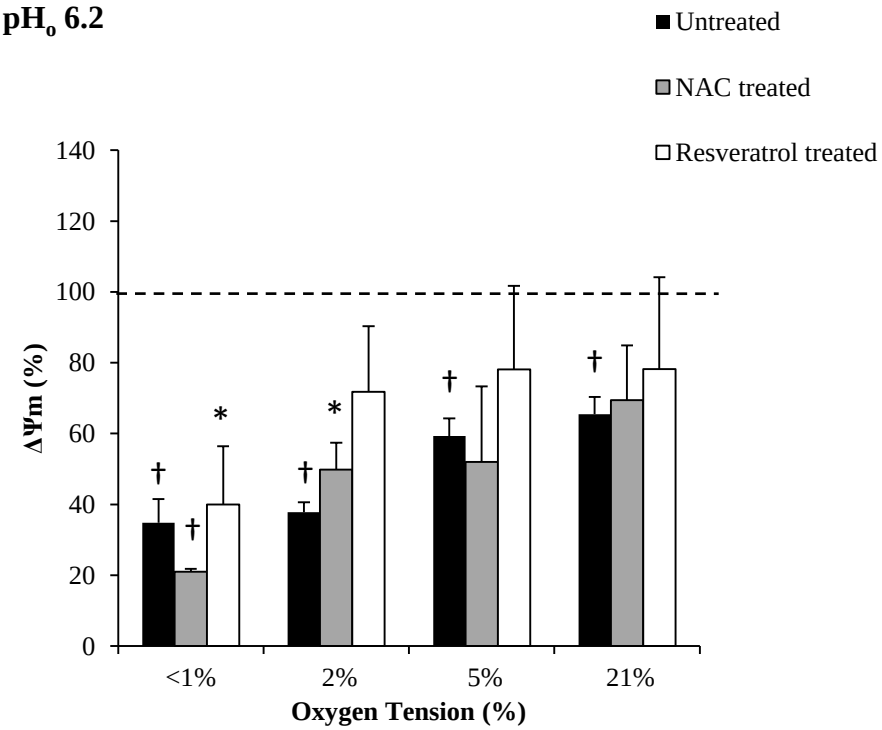
A

pH_o 7.2



B

pH_o 6.2



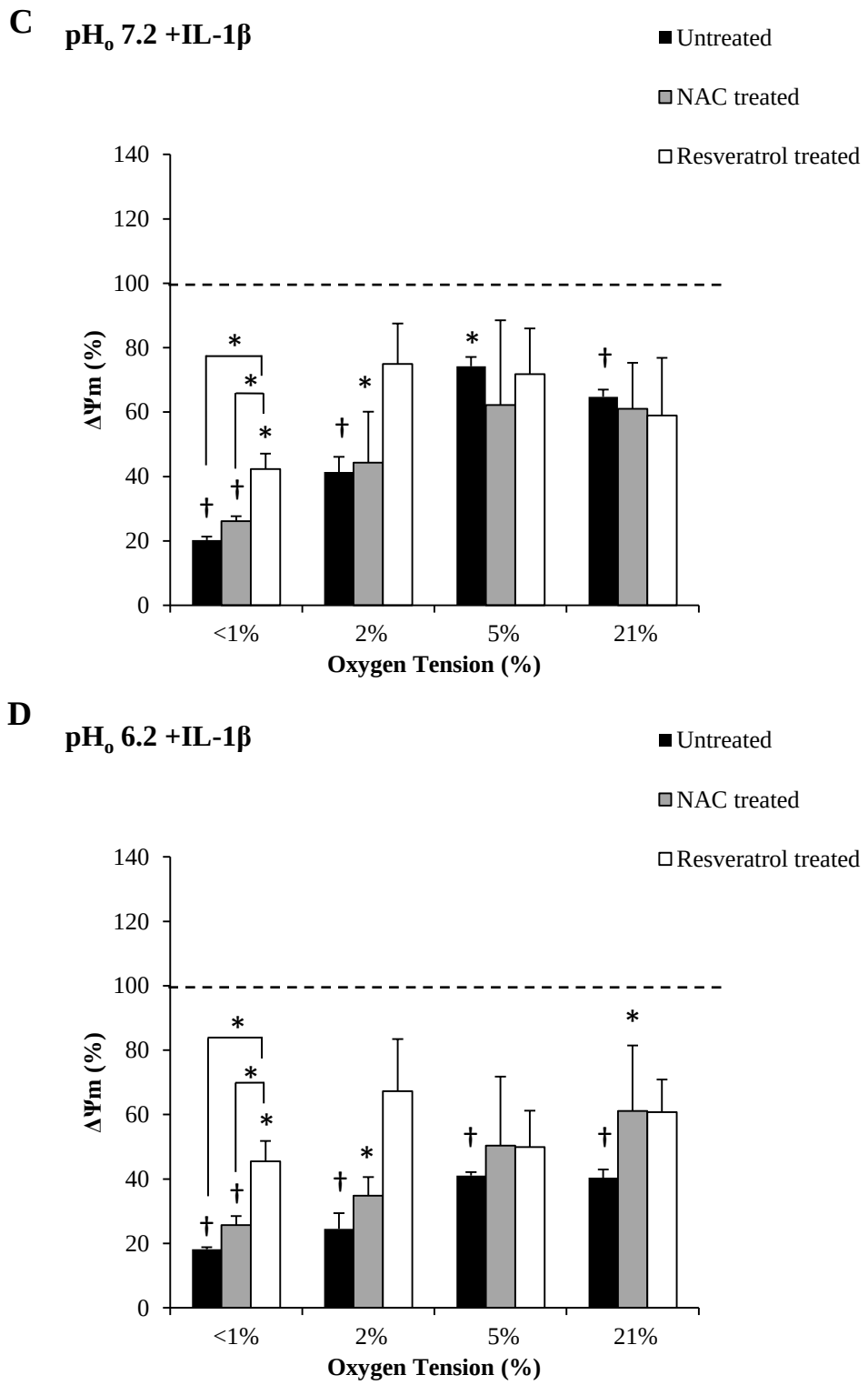


Figure 4.6. Effect of O_2, pH, IL-1β and antioxidant compounds on HOAC ΔΨm after 48 hours. ΔΨm was determined using the fluorescent probe JC-1 and results are presented as mean values compared to control (time=0, 5%O2, pH7.2HOAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O2 at pH 7.2 (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10μM) and ΔΨm was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.001 compared to control.

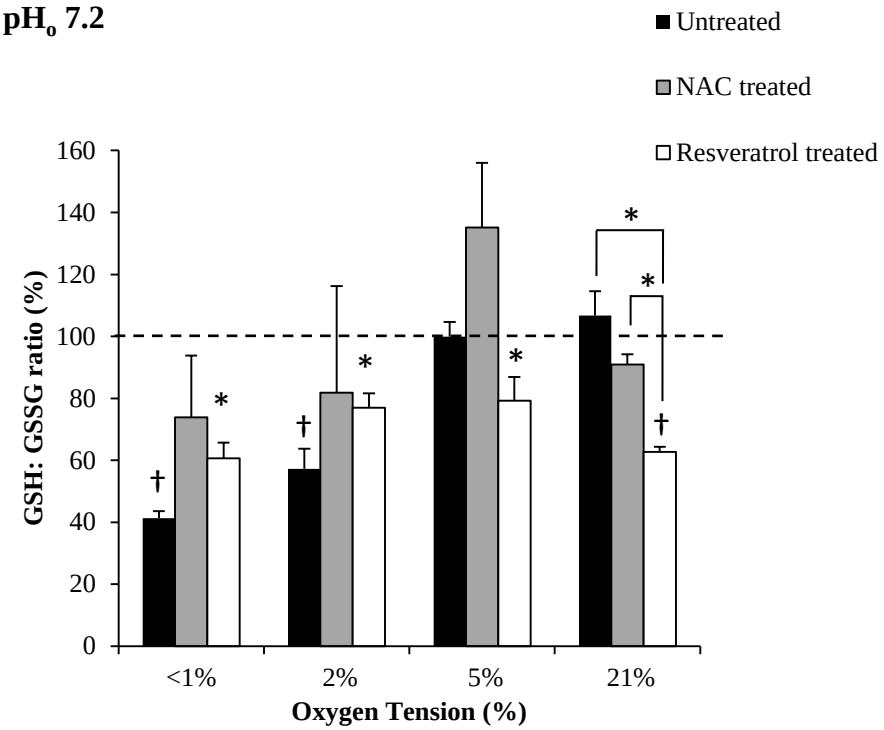
GSH:GSSG ratio

Glutathione in its reduced state (GSH) is a versatile and diverse cellular antioxidant, detoxifying ROS (specifically H_2O_2). The vast majority of glutathione exists in its reduced form (GSH) but can be readily oxidised to form GSSG (oxidised state) (Dalton et al, 2004). The ratio of GSH:GSSG is a key determinant of cellular redox status and can be used as a marker of antioxidant depletion. The effects of O_2 tension and pH on GSH:GSSG ratios in HOACs was measured in the absence or presence of IL-1β and NAC or resveratrol.

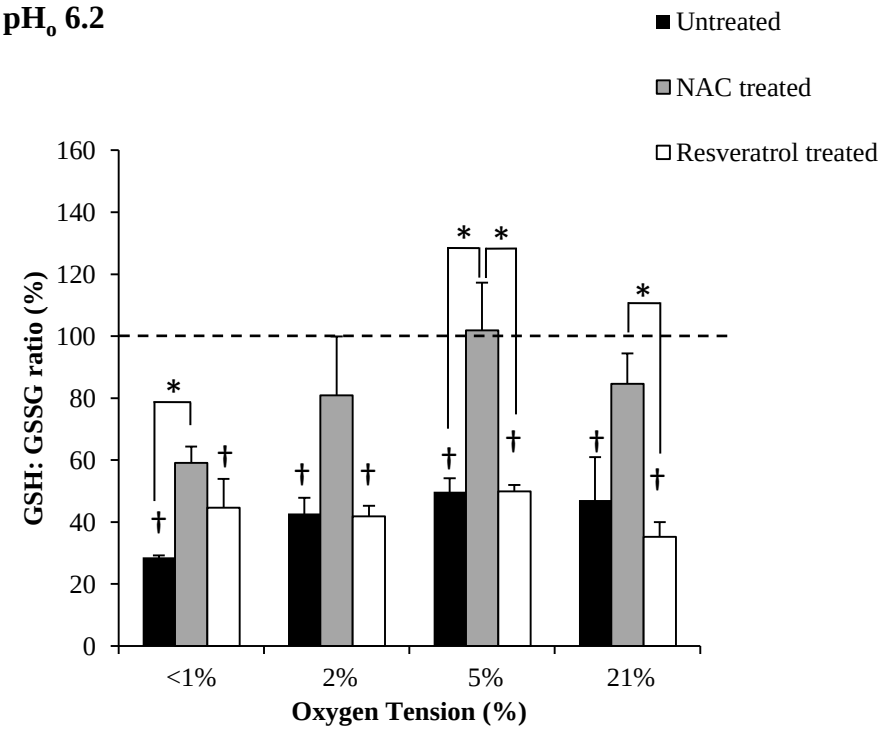
In pH 7.2 conditions, the GSH:GSSG ratio of OA chondrocytes decreased with reduced oxygen levels. Reductions to <1% and 2% O_2 led to significant reductions in GSH:GSSG ratio (41.3 ± 7.6% and 57 ± 6.4%, respectively, both P=<0.001 (figure **4.7A).** Acidosis and the presence of IL-1β markedly reduced GSH:GSSG independent of the oxygen level (figure **4.7 B–D**). With the addition of N-acetyl cysteine, GSH:GSSG levels were increased in all conditions. Of note, the majority of GSH:GSSG levels were returned to control values and significant increases in GSH:GSSG levels were seen in acidic conditions at <1% and 5% O_2 and in the presence of IL-1β at <1% and 21% O_2 (pH 7.2 and 6.2) and at 5% O_2 (pH 6.2) compared to untreated samples. In contrast however, the addition of resveratrol, had little effect on GSH:GSSG levels.

In order to calculate GSH ratios, individual measurements of both GSH (reduced glutathione) and GSSG (oxidised glutathione) had to be determined. Observed changes in the GSH:GSSG ratios were specifically caused by changes in GSH. In this study, GSSG levels appeared to be unaffected by any of the experimental conditions (figure **4.8 A-C**). Of specific note is the observation that GSH levels were highest in normal chondrocytes supplemented with NAC, with the GSH ratio peaking at a ratio of 8:1 (in 5% O_2, pH7.2 conditions) (figure **4.8B**). This is in accordance with the purported antioxidant role of NAC in restoring intracellular glutathione levels.

A **pH_o 7.2**



B **pH_o 6.2**



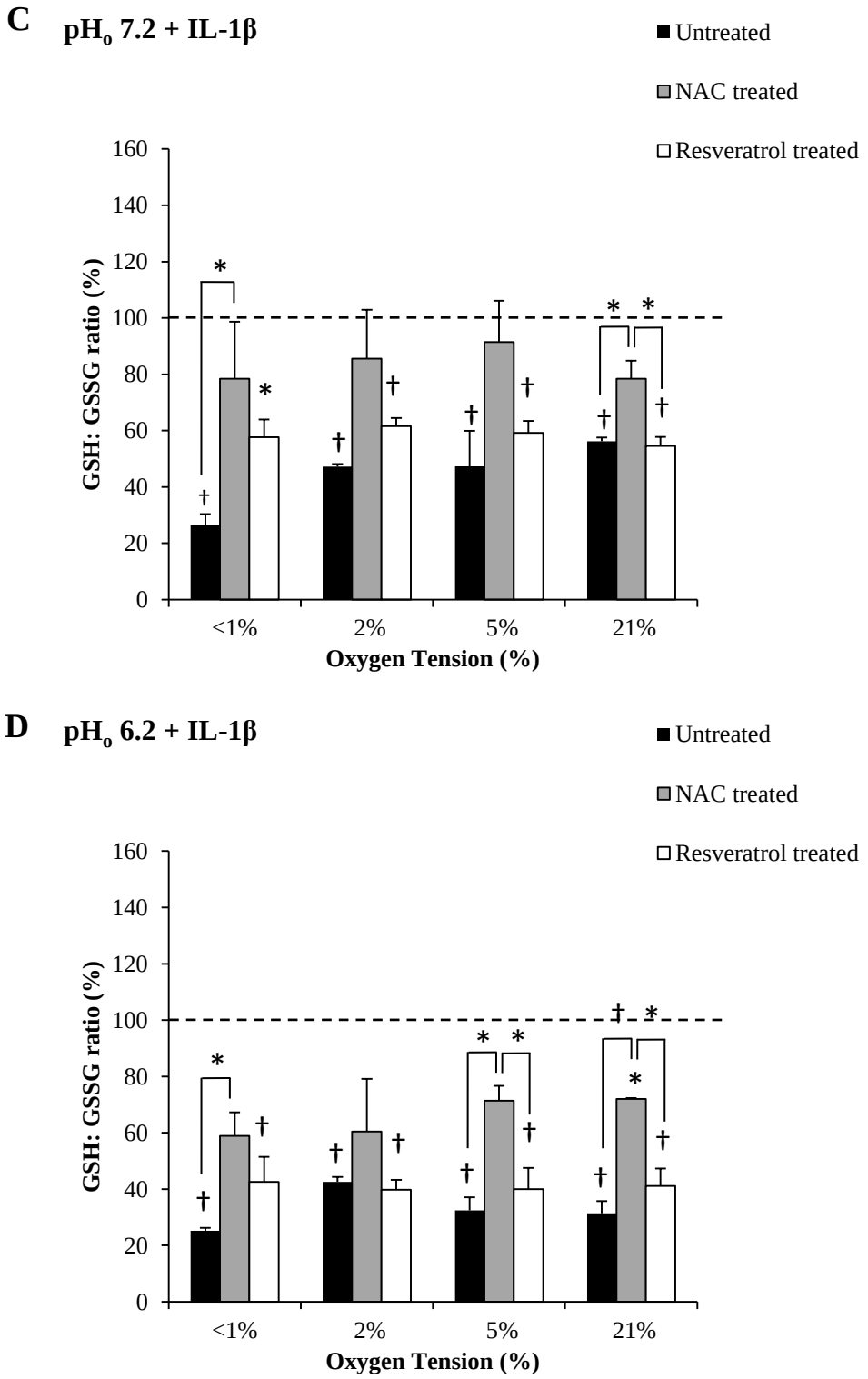


Figure 4.7. Effect of O_2, pH, IL-1β and AOX compounds on HOAC GSH:GSSG ratio. Ratios were measured using the GSH:GSSG-Glo™ Assay and results are presented as mean values versus control (time=0, 5%O2, pH7.2). HOAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O2 at pH 7.2 (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of NAC (2mM) or resveratrol (10µM) and GSH:GSSG ratio was calculated at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

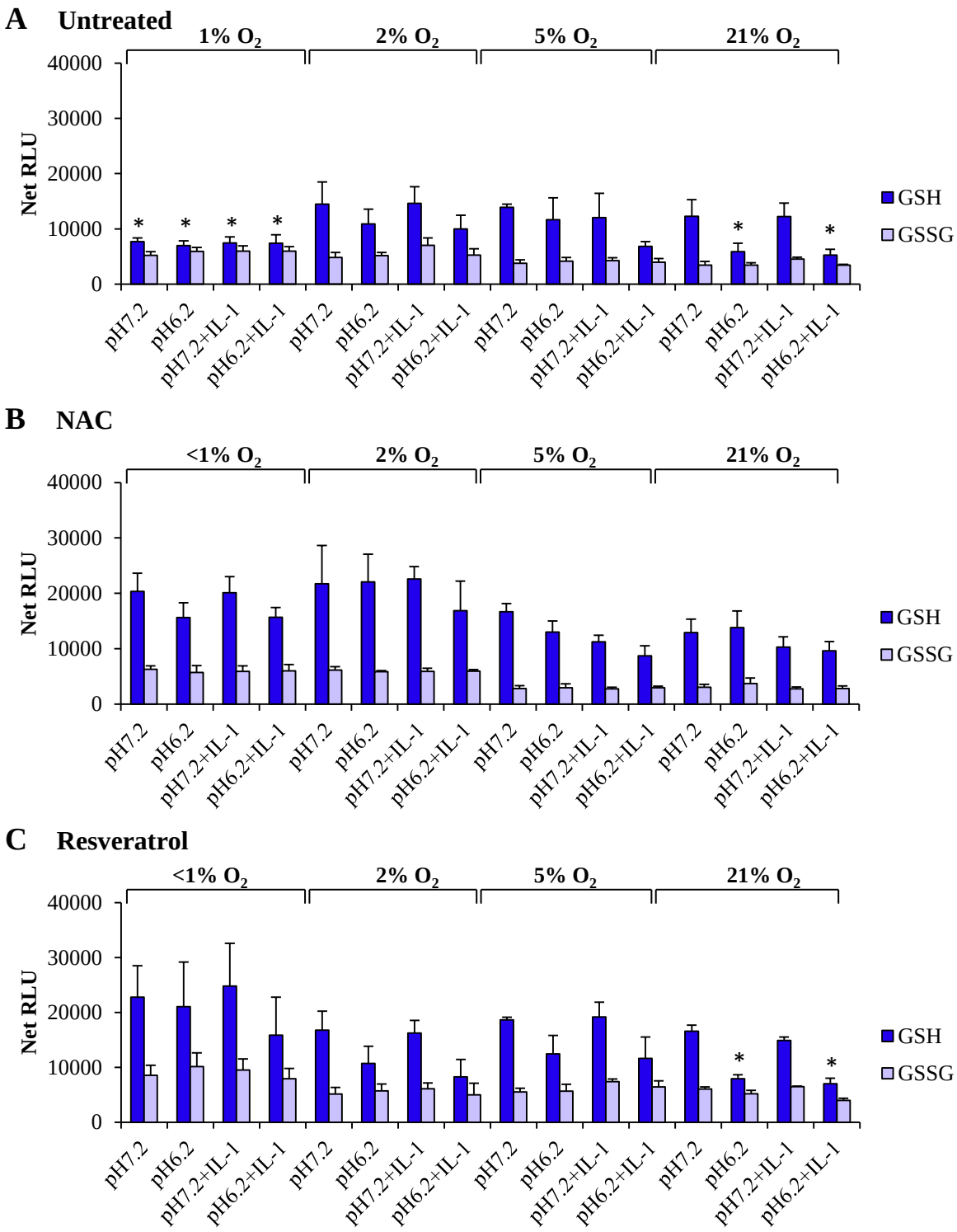


Figure 4.8. Reduced (GSH) and oxidised (GSSG) glutathione levels in articular chondrocytes. Panel **A** demonstrates the effect of O_2 tension, pH and IL-1β on GSH and GSSG levels in human osteoarthritic articular chondrocytes. Panels **B** and **C** demonstrate the effect of NAC and resveratrol on GSH and GSSG values in osteoarthritic articular chondrocytes. Bar charts represent means ± SEM, n=3donors. *P=<0.05 compared to control.

4.4.5. Intracellular pH (pH_i)

In chondrocytes, pH_i displays a close relationship with extracellular pH, both of which may be influenced by oxygen tension (Milner et al, 2006; Wilkins & Hall, 1995). To assess the effects of oxygen levels and extracellular pH on pH_i, human OA chondrocytes were incubated in different oxygen tensions and extracellular pH in the absence or presence of IL-1β for 48 hours and pH_i was measured using the pH sensitive fluorescent dye, BCECF.

At oxygen tensions above <1%, pH_i was maintained to ~pH 7.2 in all conditions. Culture of human OA chondrocytes in anoxia however led to significant reductions in pH_i. This appeared to be independent of extracellular pH or the presence of IL-1β. In anoxic conditions, pH_i was reduced to 6.85 ± 0.03 (*P*=0.018) and 6.89 ± 0.06 (*P*=0.030) in pH 7.2 and pH 6.2 conditions (table **4.2)**.

O_2 tension	pH_o 7.2	pH_o 6.2	pH_o 7.2+IL-1β	pH_o 6.2 +IL-1β
<1% O_2	**6.85** ± 0.03 *	**6.89** ± 0.06 *	**6.85** ± 0.08 *	**6.80** ± 0.11 *
2% O_2	**7.25** ± 0.13	**7.18** ± 0.05	**7.30** ± 0.17	**7.24** ± 0.20
5% O_2	**7.42** ± 0.12	**7.21** ± 0.09	**7.32** ± 0.08	**7.14** ± 0.03
21% O_2	**7.32** ± 0.06	**7.16** ± 0.06	**7.19** ± 0.02	**7.17** ± 0.03

Table 4.2. Effect of oxygen levels, pH and IL-1β stimulation on the intracellular pH (pH_i) of HOAC after 48 hours. (pH_i) was determined using the BCECF method and presented as mean pH values. Osteoarthritic chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O2 at pH 7.2, pH6.2 and in the absence or presence of 10ng/ml IL-1β for 48 hours and pH_i was measured. Table shows means ± SEM, n=3 donors. **P*=<0.05 compared to control (time-0, 5% O_2, pH 7.2).

4.4.6. Protein expression of SOD, HIF-1α, NRF2 and KEAP1

Since oxygen tension and pH had significant effect on the GSH:GSSG ratio in human chondrocytes, the next aim was to detect and quantify the expression of the antioxidant enzyme superoxide dismutase in cells exposed to a low oxygen/pH environment. Furthermore, the detection and quantification of the expression of proteins linked to oxygen sensitivity (HIF-1α) and redox status (NRF-2, KEAP1) were performed in HOAC.

Superoxide dismutase 1 and 2 (SOD1/SOD2) protein expression

Figure **4.9** shows a representative western blot of SOD1 and SOD2 expression in OA chondrocytes after exposure to different oxygen levels and pH in the absence or presence of IL-1β, NAC or resveratrol for 48 hours. The housekeeping protein, α-tubulin, was used as a loading control. Protein expression from each condition is presented in tables **4.3** (in the absence of IL-1β) and **4.4** (in the presence of IL-1β).

Expression of SOD1 was unchanged from control in AOX untreated conditions regardless of O_2 and pH levels. Similarly, the addition of NAC had no effect on SOD1. Resveratrol however, significantly increased SOD1 expression in normal and acidic conditions but only when in 5% oxygen levels. The addition of IL-1β to OA chondrocyte cultures had no effect on SOD1 expression in AOX untreated conditions but resveratrol had a significant effect in increasing SOD1 expression in HOAC in the presence of IL-1β (185.4 ± 16.6%, P=0.02) (table **4.4**). Interestingly, an effect of anoxia to reduce SOD1 protein expression appears evident in NAC treated cultures, but this effect only reached significance in acidic conditions with IL-1β stimulation (25.6 ± 6.1%, P=0.01).

SOD2 protein expression was unaffected by oxygen tension, extracellular pH and IL-1β stimulation in both AOX untreated conditions and NAC treated conditions at 48 hours. Despite modest increases in SOD2 expression with NAC supplementation the results were not statistically significant. Addition of resveratrol to OA chondrocytes however had a significant effect on SOD2 protein expression, particularly at pH 7.2

(table **4.3**). In 5% oxygen, SOD2 was increased 2.5 fold compared to control (248.1 ± 0.7, P=0.001). The effect of resveratrol on increasing SOD2 expression was maintained, despite acidosis, IL-1β stimulation or a combination of these factors. Thus, resveratrol led to a greater than 2-fold increase in SOD2 expression in acidosis and exposure to IL-1β (234.9 ± 17.5%, P<0.001) (table **4.4**).

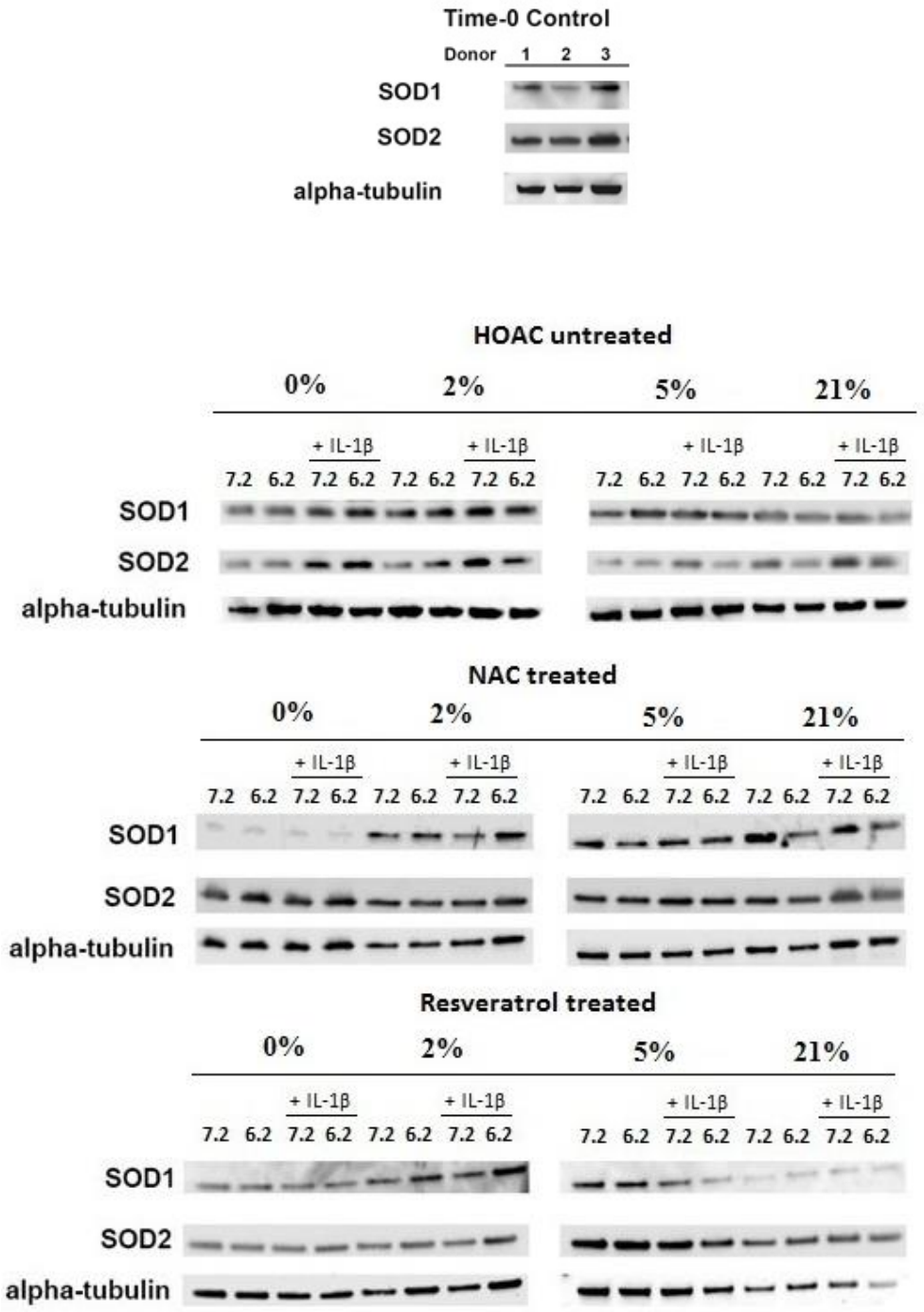


Figure 4.9. Representative western blots showing SOD1 and 2 protein expression of HOAC cultured in different O_2 tensions, pH and IL-1β in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM). HOAC (n=3) were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the absence or presence of IL-1β (10ng/ml), NAC (2mM) or resveratrol (10µM) (n=3 donors). After experimental incubation cells were released from alginate encapsulation, lysed, separated via SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and incubated in primary antibodies against SOD1 and SOD 2. Alpha-tubulin was used as a loading control.

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
Control	**5%**	**7.2**	100.0 ± 4.03	-	100.1 ± 2.09	-
UNTREATED	**<1%**	**7.2**	95.3 ± 6.2	0.972	63.9 ± 16.0	0.124
	2%		146.7 ± 8.3	0.255	64.8 ± 12.0	0.114
	5%		119.6 ± 33.1	0.890	75.9 ± 28.1	0.599
	21%		93.1 ± 29.3	0.998	78.9 ± 9.4	0.582
	<1%	**6.2**	108.1 ± 5.2	0.848	65.8 ± 10.1	0.150
	2%		169.3 ± 32.5	0.063	81.9 ± 12.5	0.567
	5%		73.2 ± 24.2	0.758	73.8 ± 12.2	0.534
	21%		78.9 ± 14.7	0.880	70.2 ± 20.9	0.316

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
NAC TREATED	**<1%**	**7.2**	92.6 ± 45.7	0.998	144.6 ± 25.1	0.636
	2%		186.3 ± 30.5	0.155	145.5 ± 12.5	0.700
	5%		157.5 ± 44.0	0.594	157.1 ± 13.2	0.203
	21%		168.5 ± 25.3	0.157	100.2 ± 46.0	1.000
	<1%	**6.2**	63.4 ± 23.8	0.690	184.8 ± 44.7	0.162
	2%		216.9 ± 22.7	0.044	154.3 ± 32.6	0.573
	5%		132.4 ± 31.1	0.901	134.8 ± 13.8	0.580
	21%		115.2 ± 28.2	0.966	121.0 ± 17.1	0.959

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
RESVERATROL TREATED	**<1%**	**7.2**	156.1 ± 13.0	0.118	170.1 ± 11.3	**0.049***
	2%		141.3 ± 32.1	0.767	185.3 ± 9.1	**0.045***
	5%		219.2 ± 30.7	**0.003***	248.1 ± 0.7	**0.001†**
	21%		165.2 ± 43.0	0.429	202.0 ± 23.2	**0.046***
	<1%	**6.2**	95.8 ± 24.4	0.999	157.8 ± 19.4	0.115
	2%		156.6 ± 48.4	0.550	156. 2 ± 31.8	0.251
	5%		208.1 ± 2.2	**0.006***	259.1 ± 20.4	**0.001†**
	21%		170.8 ± 42.8	0.364	204.5 ± 17.5	**0.049***

Table 4.3. Quantification of SOD1 and SOD2 protein expression in human OA chondrocytes. Band densitometry was used to calculate protein expression in lysates of human OA chondrocytes exposed to <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the absence or presence of NAC (2mM) or resveratrol (10µM) (n=3 donors). Values were normalised to loading control and expressed as percentages compared to control values. Table represents means ± SEM, n=3 donors. *P=<0.05 compared to control (time-0, 5% O_2, pH 7.2).

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
Control	**5%**	**7.2**	100.0 ± 4.03	-	100.1 ± 2.09	-
UNTREATED	**<1%**	**7.2+IL-1β**	116.9 ± 4.5	0.344	74.4 ± 7.0	0.335
	2%		159.4 ± 15.1	0.118	97.8 ± 13.5	0.999
	5%		78.8 ± 11.0	0.862	94.1 ± 7.0	0.994
	21%		90.5 ± 15.3	0.992	82.5 ± 9.9	0.711
	<1%	**6.2+IL-1β**	126.8 ± 12.6	0.080	81.3 ± 14.0	0.578
	2%		157.4 ± 14.5	0.134	81.5 ± 8.3	0.552
	5%		69.1 ± 12.4	0.655	90.2 ± 8.0	0.962
	21%		84.6 ± 28.0	0.955	76.0 ± 12.3	0.955

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
NAC TREATED	**<1%**	**7.2+IL-1β**	55.5 ± 18.7	0.546	170.3 ± 22.2	0.281
	2%		209.9 ± 43.7	**0.049***	174.4 ± 36.5	0.323
	5%		158.5 ± 46.6	0.581	177.4 ± 36.5	0.064
	21%		108.6 ± 7.7	0.996	111.8 ± 33.5	0.995
	<1%	**6.2+IL-1β**	25.6 ± 6.1	**0.016***	162.6 ± 26.3	0.369
	2%		191.8 ± 22.6	0.124	154.4 ± 47.3	0.571
	5%		116.9 ± 26.1	0.989	160.3 ± 39.7	0.171
	21%		113.1 ± 30.9	0.990	117.7 ± 23.2	0.977

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
RESVERATROL TREATED	**<1%**	**7.2+IL-1β**	118.2 ± 17.0	0.856	182.1 ± 23.4	**0.022***
	2%		112.0 ± 20.0	0.996	162.4 ± 15.1	0.184
	5%		185.4 ± 16.6	**0.025***	254.7 ± 19.8	**<0.001†**
	21%		148.5 ± 24.4	0.658	208.2 ± 34.7	**0.042***
	<1%	**6.2+IL-1β**	66.6 ± 18.3	0.467	166.9 ± 19.9	0.063
	2%		123.2 ± 34.5	0.956	178.0 ± 30.1	0.081
	5%		136.6 ± 20.6	0.461	234.9 ± 17.5	**<0.001†**
	21%		148.5 ± 24.4	0.993	191.1 ± 34.4	0.091

Table 4.4. Quantified SOD1 and SOD2 protein expression of human OA chondrocytes in the presence of IL-1β. Band densitometry was used to calculate protein expression in lysates of human OA chondrocytes exposed to <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the presence of IL-1β (10ng/ml), NAC (2mM) or resveratrol (10µM) (n=3 donors). Values were normalised to loading control and expressed as percentages compared to control values. Table represents means ± SEM, n=3 donors. *P=<0.05 compared to control (time-0, 5% O_2, pH 7.2).

HIF-1α protein expression

Many tissues respond to hypoxia by stabilising the highly conserved hypoxia-inducible factor (HIF). HIFs can control signalling events through the modulation of hypoxic response elements (HRE) and are widely recognised as a key regulator of the cellular response to hypoxia. HIF stabilisation is oxygen dependent and under normal oxygen levels, HIFs are rapidly hydroxylated (Husa et al, 2010; Murphy, 2010). Evidence suggests HIF-1α expression may be altered in OA tissues (Giatromanolaki et al, 2003; Yudoh et al, 2005).

Figure **4.10** shows a representative western blot (from one donor) of HIF-1α protein expression in OA chondrocytes. The control samples (time=0, 5% O_2, pH 7.2) demonstrated a lack of HIF expression, whereas in anoxic (<1%) and hypoxic (2%) conditions, HIF-1α protein expression was observed, an effect independent of pH and IL-1β exposure. This O_2 dependent effect appeared maximal in 2% oxygen conditions. HIF-1α expression was absent in higher oxygen tensions (5% and 21% O_2).

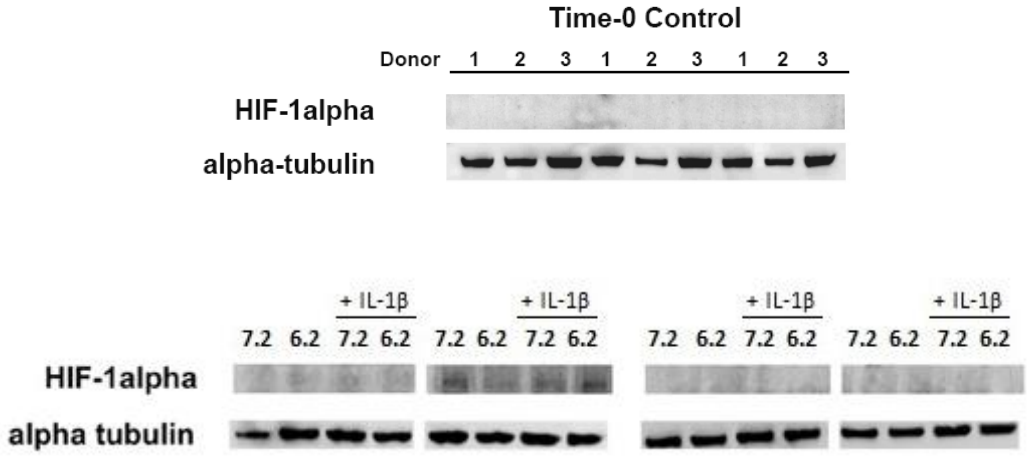


Figure 4.10. Representative western blot showing the effect of different O_2 levels, pH and IL-1β on HIF-1α protein expression in HOAC. HOAC (n=3) were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the absence or presence of IL-1β (10ng/ml). After experimental incubation cells were released from alginate encapsulation, lysed, separated via SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and incubated in a primary antibody against HIF-1α. Alpha-tubulin was used as a loading control.

KEAP-1 and NRF-2 protein expression

NRF2-mediated regulation of cellular antioxidant systems plays a key role in protection against oxidative stress in many tissues (Stepkowski and Kruszewski, 2011). The effect of oxygen (2%, 5% and 21% O_2) on NRF-2 and KEAP1 protein expression in OA chondrocytes and the effect of NAC to modulate KEAP1 and NRF2 protein expression were investigated. Data is expressed as the NRF2: KEAP1 ratio in accordance with other studies (Kanzaki et al, 2013).

Figure **4.11** shows expression of NRF2 and KEAP1 protein in human OA chondrocytes exposed to different oxygen levels in the absence or presence of NAC for 48 hours. For each oxygen condition, NRF2:KEAP1 was determined (figure **4.12**). At 5% and 21% O_2, NRF2: KEAP1 ratios were unchanged from control values. Similarly, the addition of NAC had no effect on NRF2:KEAP1 in human OA chondrocytes at these oxygen levels, although non-significant reductions occurred. Reducing the oxygen level to 2% O_2 however led to significant reductions in the NRF2:KEAP1 ratio. In AOX untreated samples, hypoxia (2% O_2) reduced NRF2:KEAP1 to 53.6 ± 12.0% of control values (P=0.032). Supplementation with NAC did not significantly alter NRF2:KEAP1 levels in hypoxia compared to AOX untreated values (39.9 ± 6.2% P=0.003).

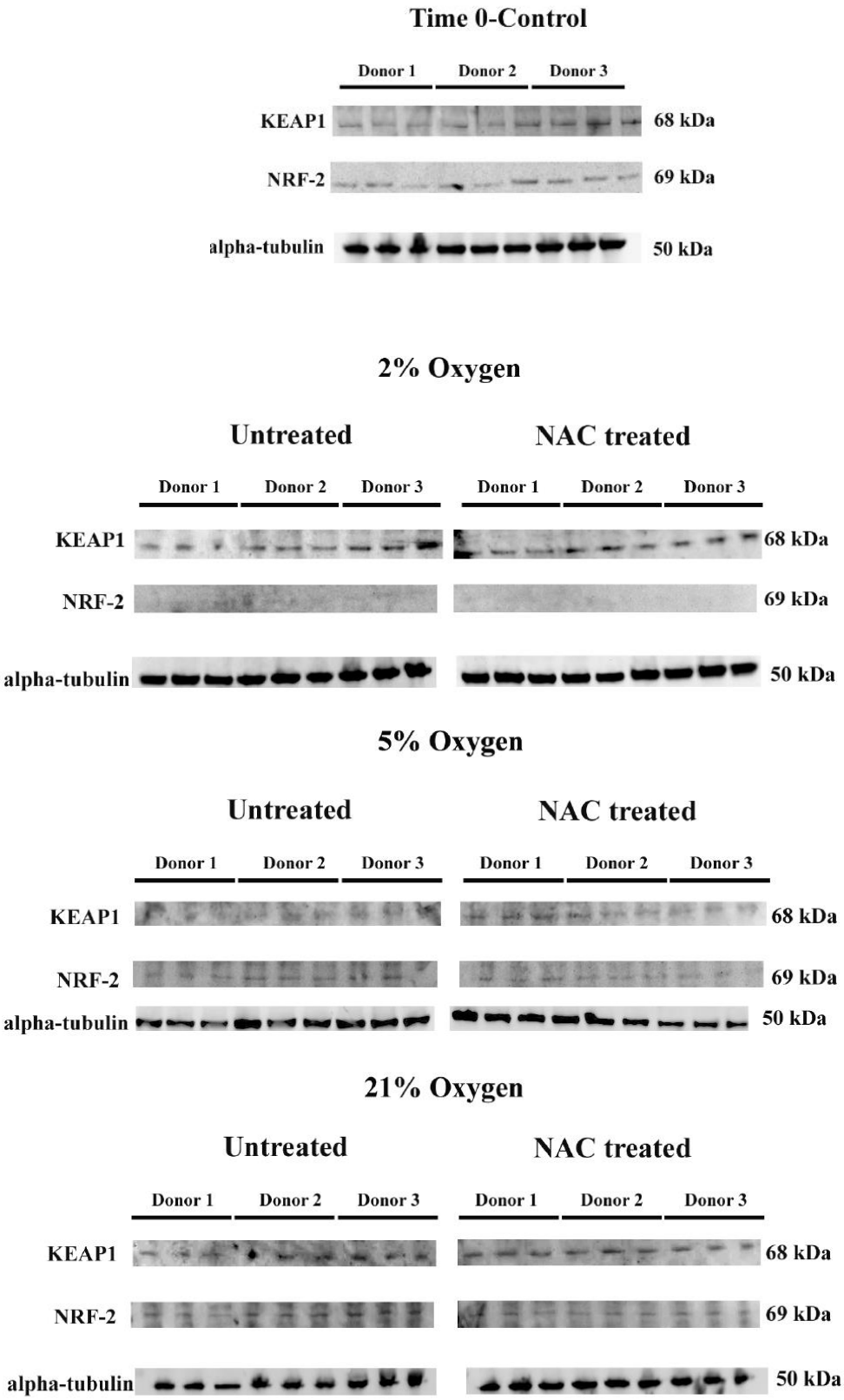


Figure 4.11. The effect of different O_2 tensions and NAC treatment on KEAP1 and NRF-2 protein expression in HOAC (n=3). HOAC were incubated in 2%, 5% or 21% O_2 in normal (pH 7.2) media for 48 hours (n=3) and expression of KEAP1 and NRF-2 was identified from cell lysates. α-tubulin was used as a loading control.

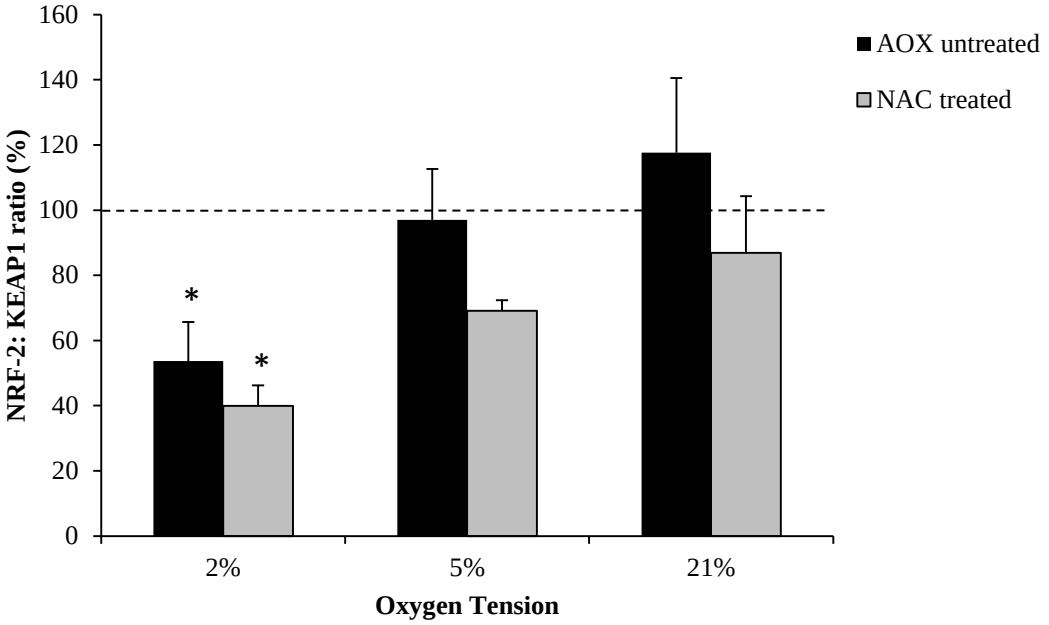


Figure 4.12. Effect of different oxygen levels and NAC treatment on the NRF-2: KEAP1 ratio of OA chondrocytes. HOAC were incubated in 2%, 5% or 21% O_2 in normal (pH 7.2) media for 48 hours (n=3) and expression of KEAP1 and NRF-2 was identified from cell lysates. α-tubulin was used as a loading control. Band densitometry was used to quantify protein expression and was normalised to control values and presented as percentage compared to control (t=0). *P=<0.05 compared to control (time-0, 5% O_2, pH 7.2).

4.5. Discussion.

The previous chapter detailed the development of a 3-D *in vitro* chondrocyte model, centred around oxygen and pH, to study the effects of changes in these environmental parameters on human OA chondrocyte function. The variations in the environmental conditions chosen are likely to mimic conditions that occur in healthy and diseased joints. It was demonstrated that deviations in oxygen levels away from the normal level of 5% O_2, and acidosis, significantly modulated chondrocyte function at 48 hours. As a result, redox related events were analysed in human OA chondrocytes at this specific time point. This chapter investigated the distinct and interactive effects of varying oxygen tensions and pH (as well as cytokine stimulation) on OA chondrocyte redox status and mitochondrial function. In addition, human OA chondrocytes were supplemented with well documented antioxidants (N-acetyl cysteine (NAC) and resveratrol), to assess their ability to influence altered redox status and to determine their action in these environmental conditions. Recent evidence postulates a role for diminished mitochondrial function in OA (Blanco et al, 2011) which is intricately linked to altered ROS levels and redox pathways, all of which appear sensitive to oxygen and pH in many cell types (Gabig et al, 1979; Milner et al, 2007; Pekun et al, 2012; Reimann et al, 2011). As oxygen tensions and extracellular pH levels are perturbed during OA (Grimshaw & Mason, 2000; Levick, 1990; Svalagosta & Kiaer, 1989), it is logical to investigate the oxygen and pH-sensitive relationship between mitochondrial function, ROS generation and redox status in human OA chondrocytes.

Oxygen-sensitivity of OA chondrocyte mitochondria and redox status

The effect of 48 hour exposure to different oxygen conditions (<1%, 2.5%, 5% and 21% O_2) on ROS generation, mitochondrial membrane polarisation, pHi, GSH:GSSG and NRF2:KEAP1 ratios and HIF-1α and SOD protein expression in HOACs was assessed. In this chapter, a significant modulatory role for oxygen on all of these variables was demonstrated. Incubation of human OA chondrocytes in 5% O_2, pH 7.2 for 48 hours led to the least deviation from control values in our study. No differences in ROS generation, ΔΨm, pHi, GSH:GSSG ratios, NRF2: KEAP1

ratios or SOD expression were detected between these conditions and control values (t=0). Incubation at 21% O_2 also had little effects on these variables; however these conditions did cause an increase in oxygen consumption, which, as discussed in the previous chapter, may indicate a metabolic switch to oxidative phosphorylation in the presence of abnormally high oxygen levels, potentially leading to inappropriate chondrocyte function.

ROS overproduction has been implicated in chondrocyte apoptosis, inhibition of matrix synthesis, and exacerbation of cartilage matrix breakdown and stimulation of pro-inflammatory mediators, all of which contribute to the progression of OA (Henrotin et al, 2003). In the present study, reductions *from* 5% O_2 inhibited ROS generation in an oxygen dependent manner. The effect of hypoxia/anoxia on ROS formation in the literature yields inconsistent results and appears dependent on several factors such as the metabolic status and type of cell, the extent of hypoxia, the presence of disease or occurrence of reoxygenation (e.g. reperfusion injuries). Many of these factors are cell type specific, for instance many aerobic tissues display increased ROS generation in hypoxia (Chandel et al, 2000; Duranteau et al, 1998; Solaini et al, 2010) whereas others demonstrate ROS inhibition (Yang et al, 1995).

The mitochondrion appears to be the primary site of ROS formation in articular chondrocytes and ROS generation is linked to mitochondrial membrane polarisation. It was observed that significant mitochondrial depolarisation occurred after 48 hours in anoxia and hypoxia (<1% and 2% O_2) which is implicit with a reduction in electron flux in the mitochondrial ETC. A reduction in the number of electrons in the ETC reduces the number of random opportunities for electrons to partially reduce O_2, forming ROS, and this likely explains the hypoxia-induced reduction of ROS observed in our study. It has been previously reported that a reduction in ROS levels in very low oxygen levels (1% O_2) occurred in normal equine chondrocytes in the short term (3 hours) (Milner et al, 2007) and therefore, this study supports these findings and extends them by showing the same effect under longer term incubations (48 hours) in human OA chondrocytes.

Mitochondrial dysfunction is beginning to be viewed as a key driver of OA progression (Vaamonde-Garcia et al, 2012). Mitochondrial depolarisation, as observed in response to hypoxia and anoxia may be seen as a marker of mitochondrial dysfunction. As mitochondria govern many key cellular pathways such as calcium handling, energy synthesis (albeit minor in chondrocytes) and cellular apoptosis, mitochondrial dysfunction may compromise cellular integrity. Indeed, mitochondrial ETC complex inhibition has been previously reported in OA chondrocytes ultimately leading to mitochondrial depolarisation (Blanco et al, 2004). Furthermore, mitochondrial membrane swelling and disruption of the outer mitochondrial membrane in OA leads to an increase in pro-apoptotic machinery and release of caspases that induces cell death (Blanco et al, 2004; Maneiro et al, 2003). The finding that physiological oxygen tensions (5%) maintain $\Delta\Psi m$ whereas reductions in oxygen levels result in depolarisation, highlights the modulatory role that oxygen exerts on mitochondrial function in diseased cartilage.

Furthermore, results in this chapter demonstrate that reduction of O_2 levels to <1% caused intracellular acidification in OA chondrocytes. This effect appeared independent to extracellular pH or the presence of IL-1β. Culture in higher oxygen tensions (5% or 21% O_2) alleviated intracellular acidification and pH levels remained between pH 7.2-7.4. The effect of short term reductions in O_2 tension on intracellular pH in chondrocytes has been demonstrated previously. Very low levels of oxygen (1%) induced intracellular acidification in equine chondrocytes through inhibition of acid efflux by Na^+/H^+ exchange (NHE). Pharmacological intervention to restore ROS levels prevented acidification and alleviated the inhibition of acid extrusion, demonstrating that ROS homeostasis is necessary for proper chondrocyte function (Gibson et al, 2009; Milner et al, 2006). Sustained intracellular acidification can lead to impairment of matrix synthesis and turnover in chondrocytes (Wilkins and Hall, 1995) and is also implicated in protease release (Furlong et al, 1997), DNA fragmentation (Gottlieb et al, 1996) and cellular apoptosis in many cells types (Matsuyama et al, 2000), all of which are implicated in the pathogenesis of OA.

It was also demonstrated that significant reductions in cellular GSH:GSSG occurred in hypoxia and anoxia. As low oxygen tension reduced ROS levels, it is interesting

to speculate whether a concomitant reduction in antioxidant systems simply reflects a reduced cellular antioxidant requirement in these environments, or whether specific antioxidant inhibition is in play. In support of the latter, hypoxia has been shown to significantly inhibit superoxide dismutase expression and GSH levels in other tissues. Exposure to hypoxia for 24 hours has been observed to reduce SOD1 and SOD2 levels in both lung fibroblasts and alveolar epithelial cells (Jackson et al, 1998). Furthermore, chronic hypoxia (7 days) significantly reduces GSH:GSSG, glutathione reductase levels and SOD2 levels in rat brain vessels, enhancing redox imbalance and potentiating neurodegeneration (Carvalho et al, 2010). Thus it appears that hypoxia can exert an antioxidant inhibitory effect in several tissues, but less is known regarding articular chondrocytes, although reduction in all SOD isoform levels and GSH levels in human OA cartilage has been demonstrated (Scott et al, 2012; Regan et al, 2008). Results from the present chapter show that changes in GSH levels are primarily responsible for fluctuations in GSH:GSSG ratios, an effect particularly noticeable in anoxia. As such, elucidating the effect of low oxygen tension on GSH synthesising enzymes such as glutamate cysteine ligase or glutathione reductase could potentially uncover the modulatory role of hypoxia on GSH homeostasis and represents an avenue of future research.

It was also demonstrated that IL-1β stimulated an increase in ROS production at all oxygen levels. This was accompanied by reductions in the ΔΨm and correlates with the significantly increased NO generation observed at 48 hours in the previous chapter (in O_2 tensions above <1%). Articular chondrocytes have been shown to exhibit respiratory burst activities in a neutrophil/monocyte-like fashion in response to IL-1β exposure (Henrotin et al, 2005; Rathakrishnan et al, 1992), which significantly upregulates collagenase expression (Lo et al, 1997) and induces apoptosis *in vitro* (Yasuhara et al, 2005). Furthermore, and in agreement with the observations in this chapter, IL-1β has been shown to induce mitochondrial depolarisation in rat chondrocytes (Yasuhara et al, 2005) and IL-1β induced mitochondrial dysfunction has been shown to increase sensitivity to, and activity of, inflammatory mediators (Vaamonde-Garcia et al, 2012). Although peroxynitrite was not measured, increases in both ROS and NO generation would likely form this powerful oxidant through interactions with $O_2^{\cdot -}$. Peroxynitrite is increased in OA

cartilage and stimulates chondrocyte apoptosis, increased matrix metalloproteinase and aggrecanase expression and inhibits aggrecan and collagen type II synthesis (Del-Carlo et al, 2003; Henrotin et al, 2003; Pujol et al, 2008). IL-1β also reduced GSH:GSSG and, twinned with the upregulation of ROS and NO in these conditions, points to perturbation of redox homeostasis and a propensity towards oxidative stress.

The expression of HIF-1α in human OA chondrocytes in low oxygen tensions (<5% O_2) was demonstrated, consistent with the notion of HIF-1α stabilisation under hypoxic conditions. HIF-1α is widely recognised as the key regulator of the cellular response to hypoxia and is expressed constitutively under low O_2 tensions. In cartilage it is involved in a wide range of chondrocyte functions including chondrogenesis, cell survival, and maintenance of phenotype and matrix production (Pfander et al, 2005; Schipani et al, 2001). HIF-1α is governed by oxygen-sensing prolyl hydroxylase enzymes, which, under normoxia are activated. Hydroxylation triggers the poly-ubiquitination of HIF-1α and targets it for proteasomal degradation. Under physiological hypoxia, such that is seen in OA, hydroxylation is inhibited, HIF-1α escapes degradation, becomes stabilised and, along with HIF-1β translocates into the nucleus where it activates a battery of hypoxia response elements capable of a variety of protective functions (Coimbra et al, 2004; Gibson et al, 2008; Murphy et al., 2009; Schipani, 2005; Yudoh et al., 2005).

HIF-1α expression has been shown to be altered in OA tissues, being enhanced in areas of significant degeneration (Yudoh et al, 2005) and in OA synovial linings (compared with non-diseased tissue) (Giatromanolaki et al, 2003). Further elucidating the role of hypoxic signalling through HIF activation in OA is warranted and may lead to identification of a therapeutic target, especially in light of recent evidence suggesting a modulatory effect of ROS on HIF-1α expression in other tissues (Archer et al, 2007; Brunelle et al, 2005; Klimova et al, 2008).

Alterations in redox status and mitochondrial function by extracellular pH

The work in this chapter shows that incubation in acidosis (pH 6.2 media) is an important regulator of redox balance and mitochondrial function in human OA chondrocytes. It was found that 48 hours incubation in acidic conditions (pH 6.2) significantly compromised expression of key matrix components in human OA chondrocytes. In O_2 tensions below 21% O_2, acidosis significantly reduced COL2A1 protein expression, whilst anoxia also significantly attenuated SOX9 expression after 48 hours. This correlated with significantly increased GAG release in anoxic conditions. These results are in accordance with others who demonstrate the catabolic effects of acidosis or hypoxia *alone* on chondrocyte phenotype and ECM components, but we extend these findings to demonstrate the dual effect of low O_2 and pH on chondrocyte ECM status and phenotype

Mitochondrially derived ROS have been shown to increase in response to extracellular acidosis in rat cancer cell lines (Reimann et al, 2011) and in rat brain synaptosomes (Pekun et al, 2012). The latter study also observed significant depolarisation in acidosis which correlated with a reduction in SOD levels causing oxidative stress. As a result, effects of acidosis on ROS generation and mitochondrial membrane polarisation in OA chondrocytes were investigated. Increased ROS levels and significant reductions in mitochondrial polarisation occurred in response to extracellular acidosis. Rong and co-workers (2012) recently demonstrated that partial restoration of mitochondrial membrane depolarisation in response to extracellular acidosis led to reduction in apoptosis in rat articular chondrocytes and alongside our work (which shows acidosis-induced redox imbalance) highlights a potential therapeutic target.

As ATP synthesis through oxidative phosphorylation is intimately linked to mitochondrial membrane potentials, the reduction in ATP_i reported in chapter 3 may be a result of acidosis induced mitochondrial depolarisation although it is to be noted that ATP is intricately regulated by many processes (Carling et al, 2012; Goldenthal et al, 2004; Harris and Das, 1991). Significant reduction in GSH:GSSG of OA chondrocytes was also demonstrated pointing to redox imbalance and implicates pH

as a regulator of this process. The combined effects of acidosis and low oxygen appear to specifically reduce GSH content in human OA chondrocytes, an effect that occurs in conjunction with mitochondrial depolarisation. The explanation for GSH inhibition in the present study is unknown but may be due to (1) inhibition of GSH transport from the cytosol to mitochondria, (2) inhibition of GSSG regeneration to GSH (by GR) or (3) a lack of cysteine to contribute to GSH synthesis.

Reductions in GSH have been reported in other cells, such as hepatocytes (Gibson et al, 2008) in response to acidification and NO may inhibit GSH reductase activity (the key enzyme in regeneration of GSH from GSSG) ultimately modifying GSH content (Goldenthal et al, 2004). As increased NO generation (chapter 3) and reductions in the GSH:GSSG ratio in acidosis were observed in this study, a regulatory role of acidosis in this process is likely. Additionally, others have shown that depletion of GSH levels reduces activity of the NHE (Lee and Urban, 1997) leading to intracellular acidification. As such this could provide a potential mechanism to link extracellular acidosis, NO generation and GSH content with intracellular acidification in OA chondrocytes and indicates that further work is required in this area.

Antioxidants alter acidosis and oxygen induced redox imbalance and mitochondrial dysfunction in OA chondrocytes

Current treatment for OA is palliative and targets symptom reduction and maintenance of joint mobility and function. Supplementation with antioxidant compounds or nutraceuticals that target oxidative stress aiming to restore redox balance may be beneficial. The development of antioxidant therapy has grown in recent years but has yielded inconsistent results and is linked with controversy over efficacy of some compounds. In this chapter the effects of two well documented compounds, N-acetyl cysteine (NAC) and resveratrol on human OA chondrocytes was investigated. The concentrations of NAC (2mM) and resveratrol (10µM) used in this study have been shown by others to display significant antioxidant effects in both chondrocytes (Dave et al 2008; Dycus et al, 2013; Nakagawa et al, 2009) and other cell types (Shin et al, 2009; Roman-Blas et al, 2009). The effects of these

antioxidants under the incubation conditions in this study however have not been previously reported.

The antioxidant abilities of N-acetyl cysteine (NAC)

N-acetyl cysteine (NAC) is a cell permeable derivative of cysteine (the rate limiting substrate in GSH synthesis). The postulated antioxidant capacity of NAC is wide ranging and includes, direct ROS scavenging (Ueno et al, 2010), protection against apoptosis (Nakagawa et al, 2009), maintenance of ECM integrity and inhibition of catabolic mediators (Roman-Blas et al, 2009) but its primary role appears to be replenishment of cysteine and contribution to GSH synthesis. As a result, NAC plays a key role in preservation of GSH levels allowing efficient detoxification of H_2O_2 and maintenance of redox balance under conditions of GSH depletion (Atkuri et al, 2007; Ueno et al, 2011).

The antioxidant properties of NAC were varied in the present study but the most significant effects were related to maintenance and stabilisation of GSH:GSSG ratios. Treatment with NAC significantly reduced GAG release in 5% O_2 and also abolished acidosis and IL-1β-induced mitochondrial depolarisation. The primary finding of this part of the study however is that treatment with NAC maintains the GSH:GSSG ratio regardless of low oxygen tension or acidosis and abrogates the effects of IL-1β treatment, which, in AOX untreated conditions led to significant reductions in GSH:GSSG. NAC specifically modulated GSH:GSSG by increasing cellular GSH stores which is consistent with its mode of function.

GSH is synthesised in the cytosol from glutamate, cysteine and glycine and is dependent on the efficiency of two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH-synthetase. Thus, inhibitors of GCS or cysteine uptake have the ability to abrogate normal GSH homeostasis leading to the observed GSH deficiency seen in many pathological conditions. Conversely, growth factors or precursors of cysteine that increase cysteine stores have the ability to modulate GSH synthesis (Wu et al, 2004). The most widely used agents to maintain cysteine stores is NAC, being less toxic than cysteine itself and being well tolerated in human patients and

animals. Although direct scavenging of ROS is reported, its main role in redox homeostasis is via cysteine replenishment for GSH synthesis (Atkuri et al, 2007). As a large proportion of oxidative stress (as ROS) is formed in the mitochondrion, a requirement for mitochondrial GSH is paramount and mitochondrial GSH deficiency leads to severe mitochondrial damage in many cell types (Martensson et al, 1990).

Mitochondria lack functional GCS and GSH synthetase enzymes and are therefore reliant on membrane bound GSH transporters to sequester GSH (Fernandez-Checa et al, 1997). To date, the inner mitochondrial membrane anion transporters DIC and OGC have been shown to transport GSH into renal and hepatic mitochondria but the existence of others are debated and are currently unidentified (Lash, 2006). DIC and OGC have not been identified in chondrocytes suggesting further work is required in this area. The finding that addition of NAC abolishes mitochondrial depolarisation and maintains GSH:GSSG through increased GSH in the present study implies replenishment of GSH synthesis through enhancement of the cysteine pool and points to a therapeutic target involving $\Delta\Psi m$ stabilisation.

The antioxidant effects of NAC treatment on OA tissues has been demonstrated by others. In cultured OA synoviocytes, NAC treatment was shown to significantly reduce catabolic mediators (COX-2, PGE2, MMP-13) in response to IL-1β and significantly abolished NO production (Roman-Blas et al, 2009). Additionally, Nakagawa and co-workers (2009) observed a significant effect of NAC to reduce OA chondrocyte apoptosis, ROS production, caspase activation and cartilage destruction and along with the current findings shows the antioxidant potential of NAC in OA chondrocytes.

The antioxidant abilities of resveratrol

Resveratrol is a naturally occurring polyphenol present in grape skin, red wine, berries and peanuts and has postulated antioxidant properties in osteoarthritis by reducing cellular apoptosis, inhibiting matrix metalloproteinase release (Shakibaei et al, 2008), maintaining ECM integrity (Elmali et al, 2005) and positively modulating mitochondrial function (Dave et al, 2008). Resveratrol has also been shown to

upregulate SOD and GPx (Spanier et al, 2009), scavenge ROS and maintain ΔΨm (Shin et al, 2009). The role of resveratrol on redox balance and mitochondrial function in OA chondrocytes is yet to be fully elucidated.

The findings in this chapter demonstrate that resveratrol abrogated ROS production, partially or totally restored membrane polarisation and significantly increased SOD2 protein expression in OA chondrocytes. Several observations have demonstrated the effects of resveratrol to increase survival and lifespan, which has accelerated the study of resveratrol as a potential therapeutic agent. The work of Howitz et al (2003) demonstrated that resveratrol extended the lifespan of budding yeast by 70% and increased DNA stabilisation through activation of Sirtuins, a class of deacetylase proteins that can modulate apoptotic pathways. Similar findings on longevity, from the same research group were also demonstrated on nematode worms and fruitflies (Wood et al, 2004) and in 2006, resveratrol was shown to increase the survival of mice fed a high calorie diet through increasing AMPK activity, insulin sensitivity and mitochondrial number (Baur et al, 2006). The interest in resveratrol as a ROS regulatory agent has increased rapidly since its association with the 'French paradox' whereby a moderate intake of red wine (containing high levels of resveratrol) diminished the incidence of cardiovascular disease in the presence of a high fat diet (Borriello et al, 2010). Antioxidant abilities of resveratrol have since been observed as anti-carcinogenic, anti-inflammatory and ROS-regulatory and are implicated in many diseases including obesity, hypertension, CVD, atherosclerosis, ischemic heart disease, diabetes and cancer (Li et al, 2012). Recent evidence demonstrates the antioxidant potential of resveratrol on enhancing or maintaining mitochondrial function in many cell types (Dave et al, 2009; Henrotin et al, 2011; Shen et al, 2012; Sin et al, 2009; Zini et al, 1999).

Attenuation of hypoxic, acidic and IL-1β-induced increases in ROS generation by resveratrol occurred in human OA chondrocytes. Resveratrol induced inhibition of ROS production has been previously documented in hepatocyte and rat brain mitochondria (Shin et al, 2009; Zini et al, 1999) and may be a consequence of enhanced mitochondrial ROS scavenging by resveratrol or inhibition of mitochondrial ETC complexes (as observed by Zini et al, 1999). Results in the

present chapter do not lend themselves to ETC inhibition as it was observed that partial restoration of mitochondrial polarisation occurred with resveratrol, indicative of maintained electron flux through the ETC. Indeed, resveratrol prevented acidosis and IL-1β-mediated mitochondrial depolarisation in oxygen tensions above anoxia demonstrating both the antioxidant potential of resveratrol and the potentially damaging effect that anoxia exerts on chondrocyte mitochondria. Furthermore, GAG release was inhibited and LDH release reduced in this study. These results are in accordance with the work of others who demonstrate the protective effect of resveratrol on mitochondrial polarisation, proteoglycan integrity and apoptotic events in OA chondrocytes and other cell types (Dave et al, 2009; Shin et al, 2009).

The present study also demonstrated the effect of resveratrol to modulate SOD protein expression. A significant increase in mitochondrial SOD2 expression was measured when cells were incubated with resveratrol. This effect was particularly noticeable in 5% O_2 conditions where protein expression was significantly increased even in the presence of acidosis and IL-1β and may account for the significant attenuation of ROS.

Modulation of the NRF2 redox pathway in human OA chondrocytes by oxygen

Detection of cellular redox status is mediated in part through the transcription factor NF-E2-related factor 2 (NRF2) which displays ROS sensitivity. The activity of NRF2 is regulated in part by the cytoplasmic scaffolding protein KEAP1 which binds NRF2 and marks it for ubiquitylation and degradation by proteasomes under normal physiological environments. KEAP1 exists as a dimer and binds NRF2 in a 'latch and hinge' fashion at two motifs, the first motif binds strongly (hinge) and the second motif binds weakly (latch). Under conditions of oxidative stress the 'latch' on KEAP1 is loosened through ROS induced degradation, altering the NRF2/KEAP1 protein complex conformation (Stepkowski et al, 2011). In this new configuration, NRF2 escapes ubiquitination, stabilises and translocates into the nucleus where it accumulates and transactivates antioxidant response elements (ARE), leading to upregulation of cytoprotective pathways (Singh et al, 2010). As NRF2/KEAP1 binding responds to oxidative stress, this complex acts as a potential redox sensor

through sensitivity to ROS. Since sensitivity of ROS to oxygen tension was demonstrated, the effect of varying oxygen tensions on the NRF2:KEAP1 ratio in OA chondrocytes was analysed.

This study is the first to demonstrate protein expression of NRF2 and KEAP1 in human OA chondrocytes. Hypoxia significantly reduced the NRF2:KEAP1 ratio of OA chondrocytes. Indeed, NRF2 protein expression was not identified in hypoxic conditions whereas KEAP1 expression was increased compared to control. No effect of physiological or hyperphysiological oxygen tensions, or the addition of NAC were found in this study. A reduction in the NRF2:KEAP1 ratio is indicative of increased NRF2 degradation, as evidenced by a lack of protein expression in hypoxia. In the present study, hypoxia represents a key condition influencing OA chondrocyte responses (inhibition of ATPi, mitochondrial depolarisation, NO formation) associated with OA pathology. Hypoxia mediated inhibition of antioxidant pathways such as the NRF2 system could potentially exacerbate these responses and intensify disease progression. Conversely, as a reduction in ROS levels in hypoxia is evident, the reduction in NRF2 expression may simply reflect efficient degradation of NRF2 by KEAP1 in the absence of ROS.

The role of NRF2 and KEAP1 in chondrocyte physiology is yet to be fully elucidated and requires further work. Although in its infancy, results from this study implicate oxygen tension as a parameter capable of influencing a key antioxidant/cytoprotective pathway which represents an exciting new area of research in free radical and cartilage biology. The finding that the mitochondrial membrane protein, PGAM5 can form a ternary complex with NRF2 and KEAP1 in other tissues, tethering it to the mitochondria (Lo & Hannink, 2008), suggests that together this ternary complex may act as a potential redox sensor capable of translating mitochondrial redox signals (from oxygen /ROS/antioxidant levels) to the rest of the cell. The role of NRF2, KEAP1 and mitochondrial PGAM5 in OA and redox signalling is unknown and requires further investigation.

4.6. Conclusions

To conclude this part of the study, the effects of varying oxygen tension and extracellular pH on redox status and mitochondrial function of OA chondrocytes after 48 hours were investigated. The effect of resveratrol and NAC to alter redox and mitochondrial homeostasis in the face of these environmental challenges was also assessed. Oxygen tension significantly altered ROS levels, with anoxia and hypoxia causing ROS inhibition and mitochondrial depolarisation. The NRF2:KEAP1 ratio and HIF-1α protein expression also displayed marked oxygen-sensitivity. Acidosis also significantly altered chondrocyte function by increasing ROS levels, inducing mitochondrial depolarisation and significantly inhibiting GSH. A combination of low oxygen tension and low pH was particularly damaging and lead to significant oxidative stress in OA chondrocytes. Treatment with NAC and resveratrol altered these variables and our evidence suggests they exert distinct antioxidant properties in the face of oxygen or acid-mediated stress. NAC increased GSH levels and partially maintained mitochondrial polarisation whereas resveratrol reduced ROS levels, increased SOD2 protein expression and maintained mitochondrial polarisation.

Chapter 5

Antioxidants alter acidosis and oxygen induced redox imbalance and mitochondrial dysfunction in normal equine articular chondrocytes

5.1. Introduction

Investigations so far in this project have centred on the effect of oxygen tension and pH on diseased (osteoarthritic) chondrocytes, as these environmental parameters are significantly altered in chronic OA. Results from the last chapter demonstrate a significant effect of oxygen tension and pH to alter mitochondrial function and redox balance in end stage OA chondrocytes. Chapter 4 also showed that the antioxidants NAC and resveratrol partially modulated redox pathways and mitochondrial homeostasis. It is not known, however, to what extent these observations were a result of disease or whether similar effects occur in normal tissues. In addition, environmental changes occurring in the articular joint early in the disease process itself, specifically disturbances in oxygen and pH, may result in redox dysfunction in chondrocytes that could be attenuated in the presence of antioxidants, thus providing a means of therapeutic modulation. By elucidating the modulatory role of oxygen tension, pH and inflammatory pathways (distinct and interactive) on redox and mitochondrial homeostasis in normal chondrocytes, the fundamental understanding of disease mediated changes in cartilage may be improved. To investigate whether similar changes occur in non-diseased cartilage, normal articular cartilage from a comparable species, the horse, was studied. Articular cartilage from diarthrodial joints in mammalian species share similar biochemical and biomechanical properties and the horse provides a more readily available source of non-diseased tissue. In addition, similar disease processes (osteoarthritis) occur in the horse as in humans making comparisons valid.

5.2. Aims

The aims of this part of the project were;

(1) To study the effect of varying O_2 tensions, pH and IL-1β on cellular mitochondrial function and redox balance by measuring the mitochondrial membrane potential, ROS generation, the GSH:GSSG ratio and SOD1 and 2 protein expression of normal equine articular chondrocytes after 48 hours. Chondrocyte phenotype, cell viability and GAG release were also assessed before and after 48 hour experimental culture.

(2) To analyse the ability of exogenous antioxidant (AOX) addition (resveratrol and N-acetylcysteine) to alter mitochondrial function and redox status in normal chondrocytes exposed to varying oxygen tensions, pH and IL-1β stimulation after 48 hours.

5.3. Study Design

- **Cell culture and experimental culture conditions**

Equine hyaline articular cartilage (EQHAC) was obtained from the grossly normal metacarpophalangeal joints of skeletally mature equine forelimbs obtained from an abattoir (n=18). All equine cartilage was macroscopically graded using the macroscopic grading system put forth by Little et al (2010) and were all graded as normal. Chondrocytes were liberated from cartilage matrix by standard enzymatic digestion protocols and cell viability was determined by Trypan blue exclusion and was >95% for all donors. Primary cells were then cultured directly in three-dimensional alginate beads at a density of $4x10^6$cells/ml alginate (as previously described). Alginate beads were incubated in a 5% O_2 tension in a humidified atmosphere for 14 days. Alginate beads were then incubated in pH 7.2 (normal media) or pH 6.2 (acidosis) conditions in the absence or presence of IL-1β and in the absence or presence of NAC (2mM) or resveratrol (10µM) for 48 hours, at <1%, 2%, 5% or 21% O_2 conditions. Following experimental incubation, O_2 and extracellular pH measurements of media were taken and the remaining media sample was frozen for analysis of GAG and LDH release. Alginate beads were released from their alginate matrix by depolymerisation in sodium citrate solution and immediately analysed.

- **Western blotting**

The chondrocyte phenotype was characterised by analysing COL2A1 and SOX9 protein expression. Primary antibodies against SOD1 and SOD2 were also used for analysis of antioxidant status. The house keeping gene α-tubulin was used as a loading control (see materials and methods for antibody details (table **2.1**)).

- **Cell media analysis**

The DMMB assay was used to assess the concentration of sulphated GAG in media samples. Cell viability was quantified using the Cytotoxicity Detection Kit ^{Plus} as per the manufacturer's instructions.

- **Measurement of reactive oxygen species (ROS) generation**

Intracellular ROS levels were measured using the Dichlorofluorescin (DCF) method. Chondrocytes were pelleted, washed and seeded at $1x10^6$ in pre-equilibrated sterile saline solution and then incubated with DCFH-DA and fluorescence was measured (excitation at 490nm/emission 535nm).

- **Determination of the mitochondrial membrane potential (ΔΨm)**

ΔΨm was assessed using JC-1. Chondrocytes were incubated with JC-1 for 40 minutes and then entered onto individual cover slides. Specific filters for rhodamine and flourescein were used to determine mitochondrial polarisation as described in the materials and methods.

- **Measurement of the GSH:GSSG ratio**

The GSH:GSSG ratio was determined by the GSH:GSSG-Glo™ Assay following the manufacturer's instructions. Average RLU values for GSH and GSSG were used to determine the ratio of GSH to GSSG in unknown samples.

- **Statistical analysis**

Significant differences were determined by One-way ANOVA with a Dunnett's and Tukey HSD *post-hoc* correction. A minimal significance level of P=<0.05 was used. Unless otherwise stated, results are presented as % compared to control (5% O_2, pH7.2, time = 0).

5.4. Results.

5.4.1. Analysis of chondrocyte phenotype and cell media

The ability of 14 days in alginate bead culture to maintain a chondrocyte phenotype was initially investigated in normal equine chondrocytes by analysing COL2A1 and SOX9 protein expression at 48 hours. The effect of a range of oxygen tensions (<1%, 2%, 5% or 21% O_2) and acidosis (pH 6.2) to alter COL2A1 and SOX9 expression was also investigated. Additionally, the pH of spent media and cellular oxygen consumption (from media) was determined. The effect of fluctuations in oxygen tension, pH, IL-1β stimulation and treatment with NAC or resveratrol to stimulate GAG and LDH release (marker of cell viability) into media was also measured.

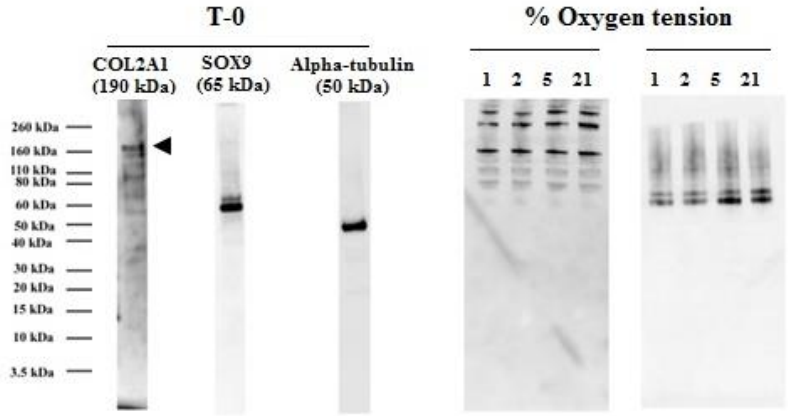
Chondrocyte phenotype

Figure **5.1A** shows that control (t=0) non-diseased equine articular chondrocytes expressed COL2A1 and SOX9 proteins after 14 days in alginate culture. COL2A1 and SOX9 protein expression was also identified after experimental incubations (t=48 hrs) in <1%, 2%, 5% or 21% O_2 tensions, in the absence or presence of acidosis. Homogeneity in protein expression was evident between conditions and band densitometry analysis showed no significant differences in protein expression compared to control in any condition studied (figure **5.1 B -C**), demonstrating no change in phenotype of equine chondrocytes following hypoxia and acidosis over this time course.

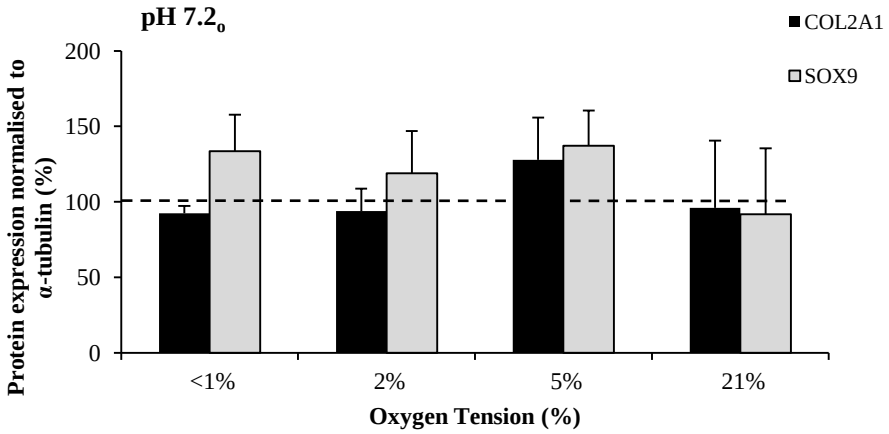
pH levels and oxygen concentration of spent media

pH levels of media were unchanged from pre-titrated starting values after 48 hours (table **5.1A**). Similarly, O_2 levels of spent media remained steady state in 2%, 5% and 21% O_2 tensions. The addition of sodium dithionite to cell media reduced the O_2 tension of anoxic cultures to 0.75 ± 0.30% and therefore, the term anoxia refers to an oxygen tension below 1% (<1% O_2) in this study (table **5.1B**).

A



B



C

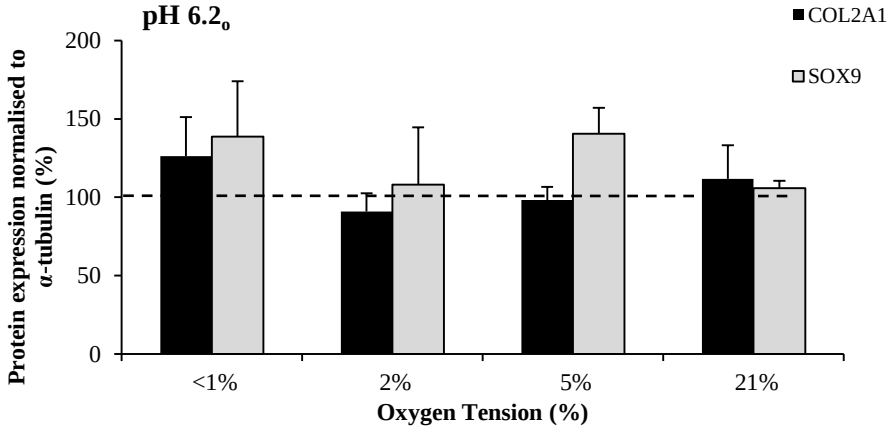


Figure 5.1. Western blotting analysis of primary normal EQHAC cultured in three-dimensional alginate beads. Western blotting for COL2A1 and SOX9 was performed on control cell lysates (t=0) (representative full lane blot) and on normal EQHAC were incubated in <1%, 2%, 5% or 21% O_2 in pH 7.2 or pH6.2 media for 48 hours. Lanes 1-4 correspond to <1%, 2%, 5% and 21% O_2 conditions (pH 7.2) (representative) (**A**). Band densitometry was used to quantify protein expression in cells cultured in pH 7.2 (**B**) and pH 6.2 (**C**) conditions. Data was normalised to α-tubulin and presented as percentage compared to control (T=0). *P=<0.05 compared to control (time-0).

A

pH levels of spent media				
O_2	**pH_o 7.2**	**pH_o 6.2**	**pH_o 7.2+IL-1β**	**pH_o 6.2 +IL-1β**
<1%	7.12 ± 0.09	6.30 ± 0.04	7.14 ± 0.12	6.31 ± 0.01
2%	7.13 ± 0.05	6.31 ± 0.04	7.11 ± 0.11	6.34 ± 0.02
5%	7.18 ± 0.05	6.32 ± 0.02	7.09 ± 0.05	7.02 ± 0.04
21%	7.12 ± 0.04	6.33 ± 0.03	7.03 ± 0.05	6.32 ± 0.02

B

O_2 tension of spent media				
	<1%	**2%**	**5%**	**21%**
Untreated	0.31 ± 0.20	1.38 ± 12	6.38 ± 0.05	19.15 ± 0.30
NAC treated	0.58 ± 0.84	3.71 ± 0.22	5.61 ± 0.63	19.99 ± 0.31
RES treated	1.34 ± 0.32	1.61 ± 0.26	4.51 ± 0.28	18.39 ± 0.24
Mean	**0.75% ± 0.3**	**2.23% ± 0.7**	**5.50% ± 0.5**	**19.17% ± 0.4**

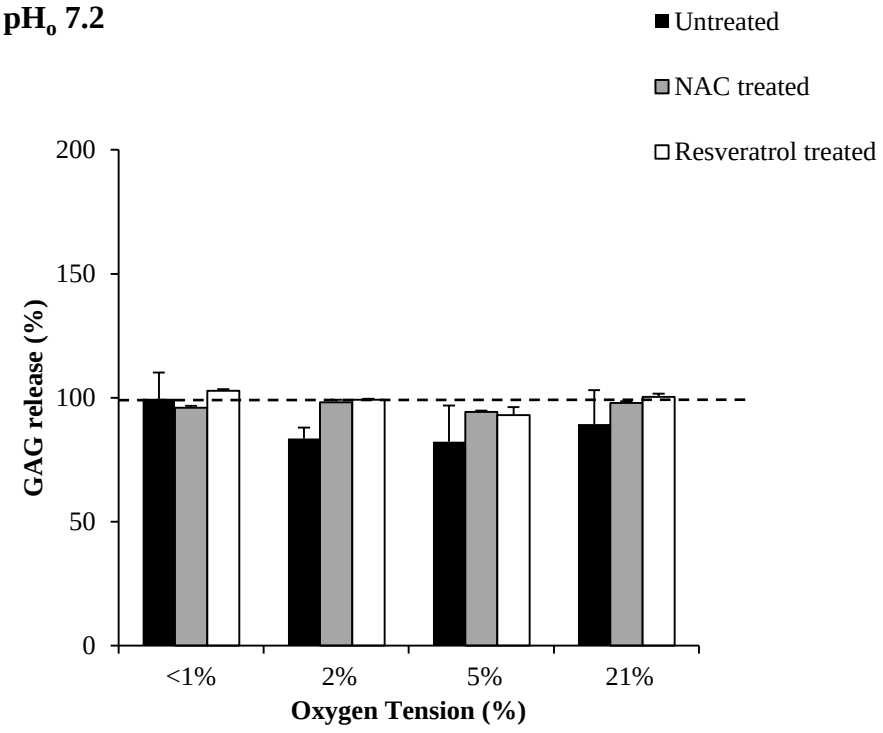
Table 5.1. Effect of oxygen, pH and IL-1β stimulation on the pH (A) and oxygen levels (B) of spent media after 48 hours. pH was determined using a pH meter and oxygen levels were measured using an oxygen electrode. Data is presented as mean pH values (n=3). Normal equine chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2, pH6.2 and in the absence or presence of 10ng/ml IL-1β for 48 hours and pH and O_2 were measured from spent culture media. Results represent mean ± SEM O_2 levels after 48 hours (n=3 donors).

Glycosaminoglycan release

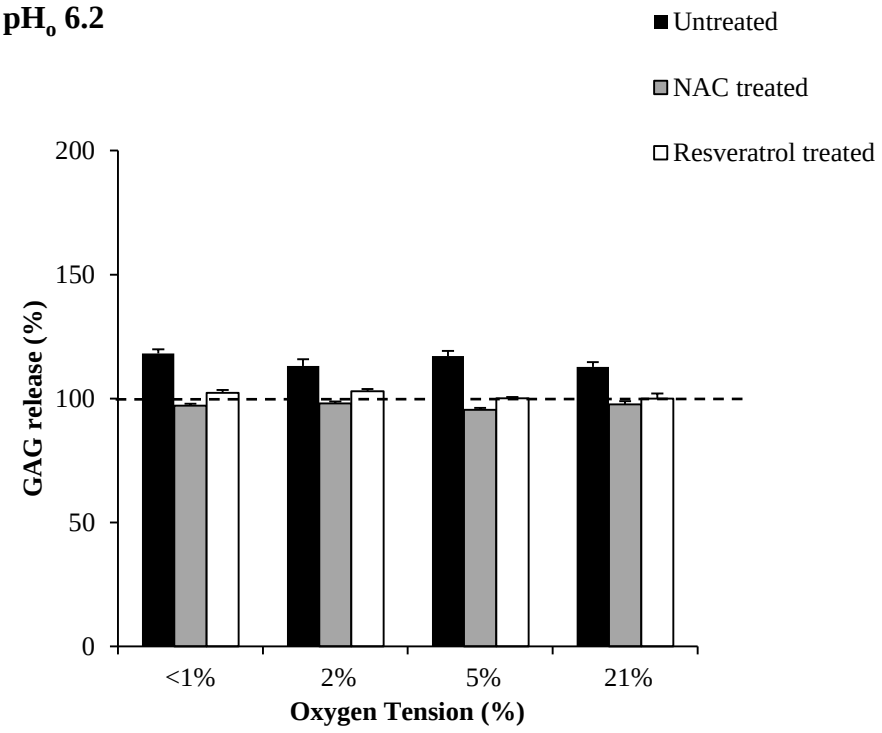
Sulphated GAG release to detect for GAG degradation products in cell culture media was determined using the DMMB method. The data demonstrate a negligible effect of O_2, pH or IL-1β to stimulate GAG release in normal equine articular chondrocytes after 48 hours incubation.

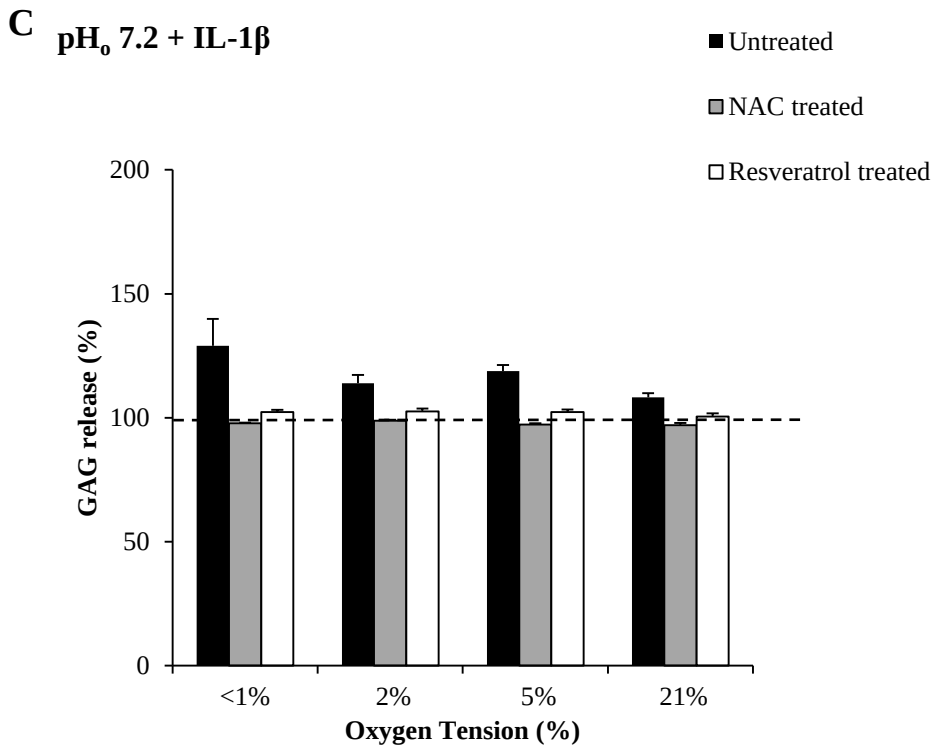
GAG release was minimal in non-diseased equine chondrocytes exposed to physiological pH conditions or acidosis (figure **5.2 A-B**). This effect was independent of O_2 tension and exogenous AOX addition. Similarly the addition of IL-1β to chondrocytes did not stimulate an increase in GAG release under physiological pH in either the absence or presence of NAC or resveratrol (Figure **5.2C**). Co-culture with IL-1β and acidosis lead to modest increases in GAG release in AOX untreated conditions, reaching significance in hypoxia (136.1 ± 8.9%, P=0.001). The addition of NAC or resveratrol to chondrocytes abrogated this effect and GAG release was maintained to control values (figure **5.2D**).

A **pH_o 7.2**



B **pH_o 6.2**





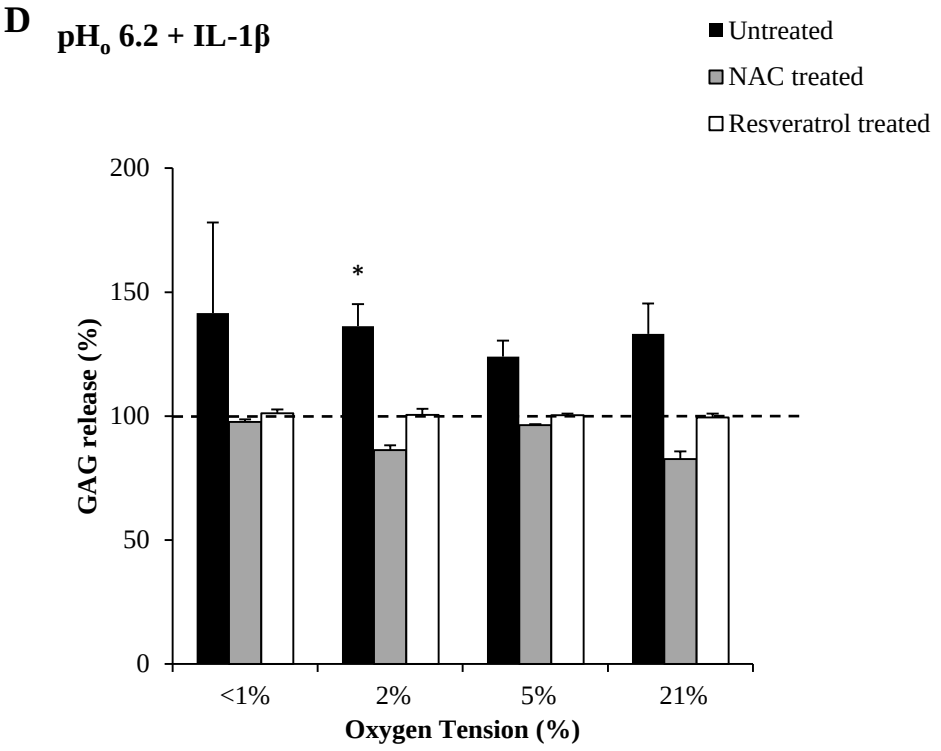


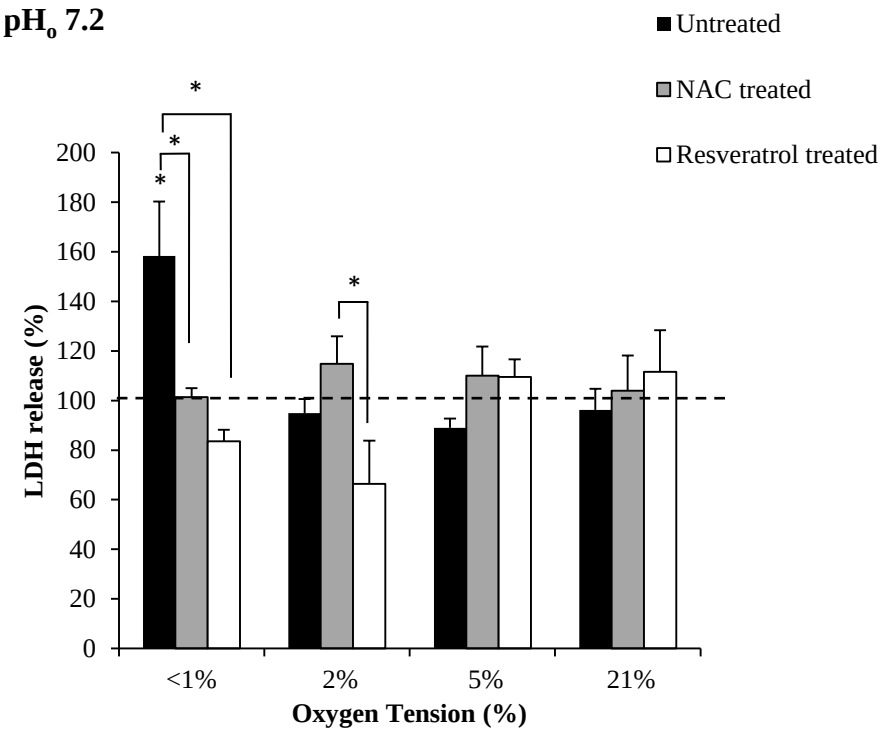
Figure 5.2. Effect of oxygen, pH, IL-1β stimulation and antioxidant compounds on EQHAC GAG release after 48 hours. GAG release was determined by the DMMB method and presented as mean values compared to control (time=0, 5%O_2, pH7.2). normal equine chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and GAG release was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01compared to control.

Cell viability

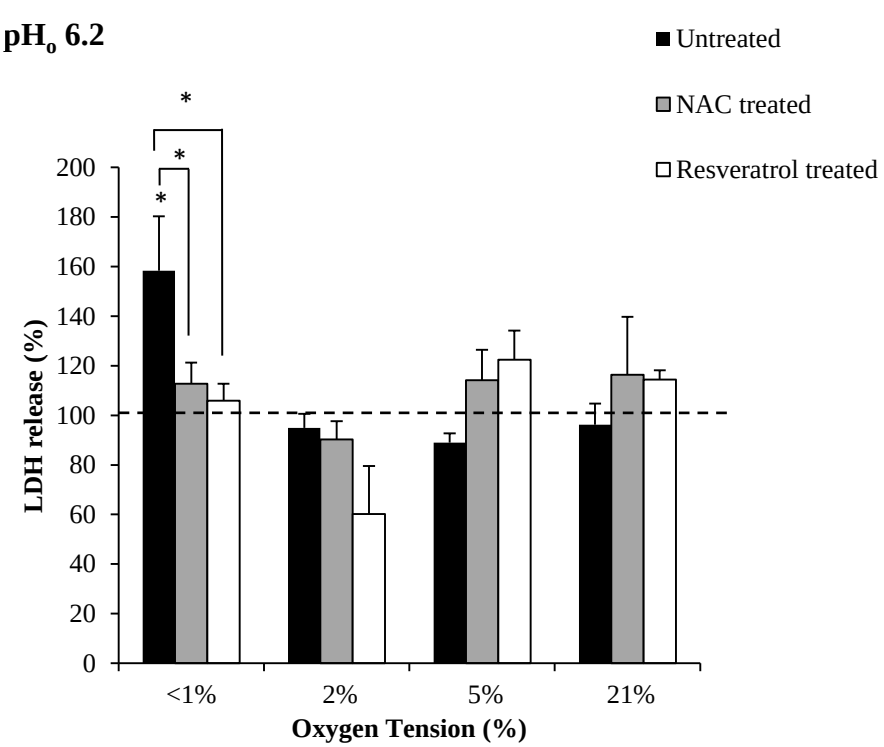
LDH release was used as a marker of cellular membrane damage and thus cell viability. Viability of normal chondrocytes was significantly modulated by anoxic conditions and exposure to IL-1β, being partially modified by exogenous AOX addition.

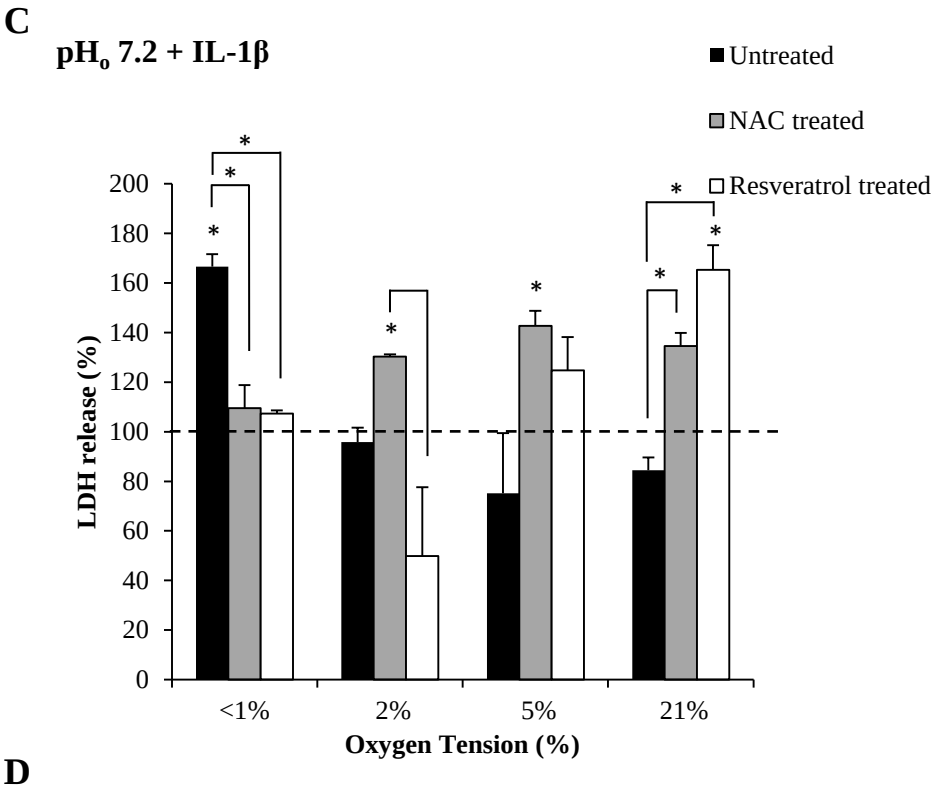
Culture in anoxic conditions led to a pH and IL-1β-independent increase in LDH release indicating reductions in cell viability at 48 hours (figure **5.3**). The addition of NAC or resveratrol abolished anoxia-induced LDH release and values were maintained to control values in every condition studied. In higher oxygen tensions (2% O_2 and above), cell viability profiles were unchanged from control in both AOX treated and AOX untreated conditions when cultured in pH 7.2 or pH 6.2 conditions (figure **5.3 A-B**). A modest effect of resveratrol to reduce LDH release in hypoxia is evident across this study but significance was not reached. AOX untreated chondrocytes were unaffected by IL-1β in O_2 tensions above <1%. Interestingly, culture in NAC and IL-1β increased LDH release in pH 7.2 conditions (figure **5.3C**). No effect of IL-1β and NAC was evident in acidotic cultures (figure **5.3D)**.

A **pH_o 7.2**



B **pH_o 6.2**





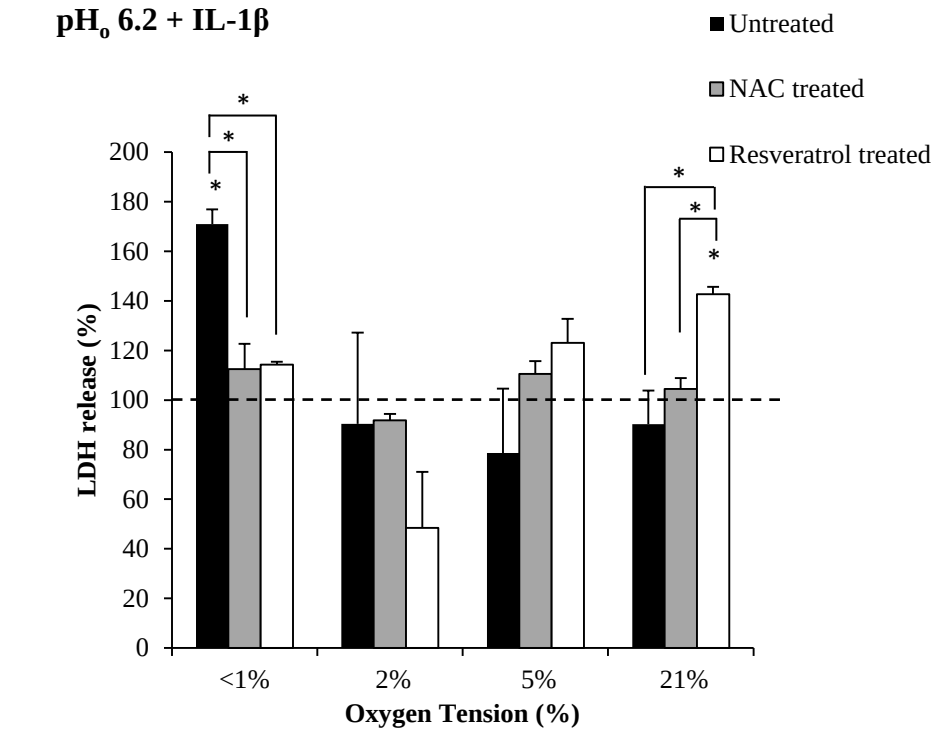


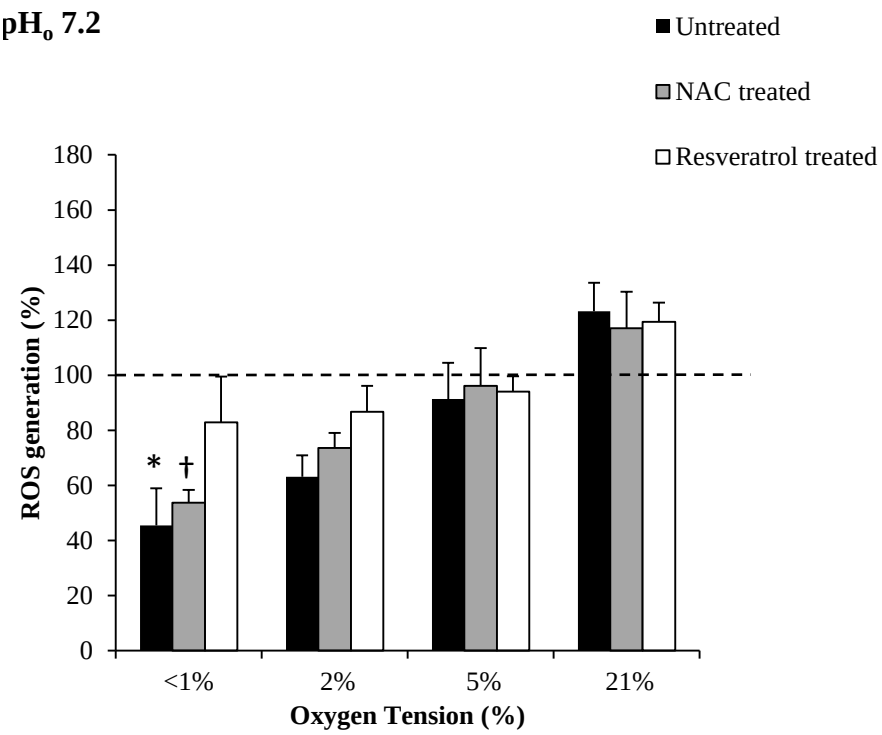
Figure 5.3. Effect of oxygen, pH, IL-1β stimulation and antioxidant compounds on EQHAC cell viability after 48 hours. Cell viability was determined by LDH release and presented as mean values compared to control (time=0, 5%O_2, pH7.2). Normal equine chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and LDH release was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01compared to control.

5.4.2. ROS levels

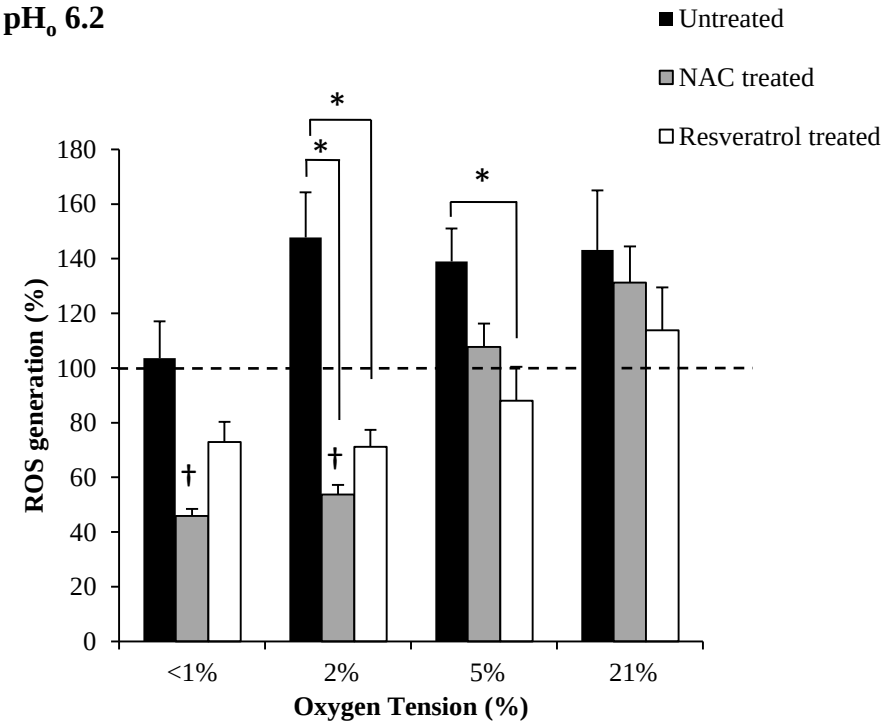
Intracellular ROS levels in normoxia (5% O_2) and hyperoxia (21% O_2) were comparable to control values after 48 hours. Reductions in oxygen tension led to reductions in ROS levels at pH 7.2. Culture in acidosis increased ROS levels but values were not significant when compared to control (figure **5.4B**). The addition of IL-1β significantly increased ROS generation in 2% and 21% O_2 tensions in pH 7.2 conditions (figure **5.4C**). The presence of IL-1β in acidic conditions significantly increased ROS levels in oxygen tensions above <1% (figure **5.4D**). Thus ROS production in normal equine chondrocytes shows oxygen and pH sensitivity, with IL-1β accentuating the increase in ROS levels observed with acidosis.

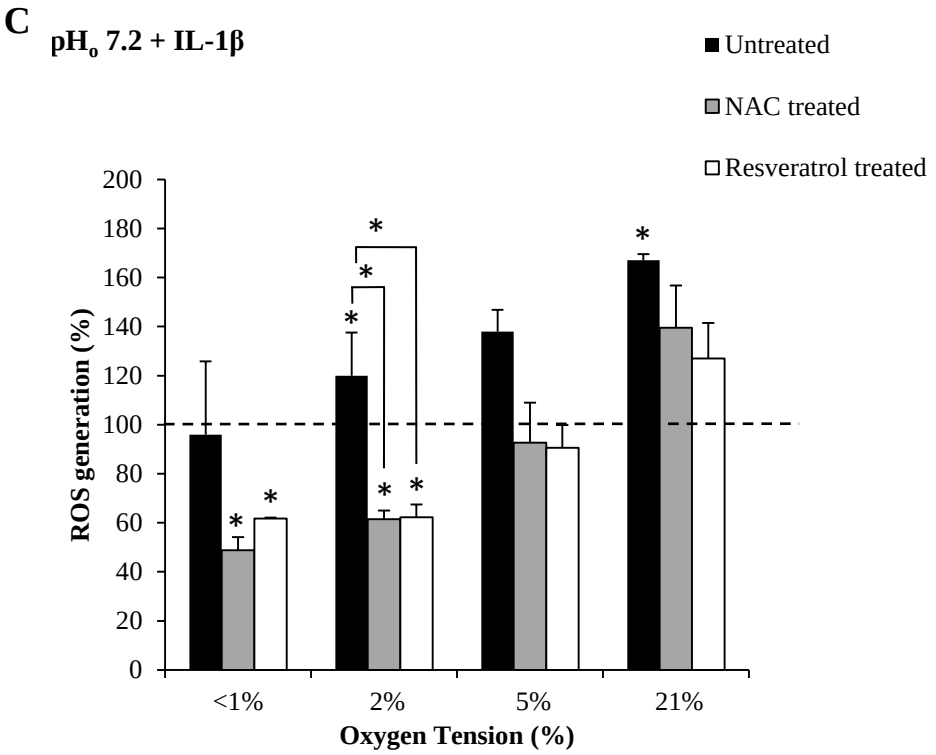
Treatment with NAC had no effect on ROS levels in pH 7.2 conditions, thus ROS production remained oxygen sensitive. Interestingly, treatment with resveratrol increased ROS levels in anoxia to values comparable to basal ROS generation (82.8 ± 16.6%, n.s), which may be particularly important when considering the role of ROS levels in redox signalling. NAC and resveratrol reduced acidosis and IL-1β-induced increases in ROS levels, particularly in <1% and 2% O_2 tensions where values were reduced to <60% of control. These results demonstrate that resveratrol abolishes hypoxic/anoxic inhibition of ROS in physiological pH, returning ROS levels to basal values. The ability of both NAC and resveratrol to suppress acid and IL-1β induced ROS release is also evident.

A **pH_o 7.2**



B **pH_o 6.2**





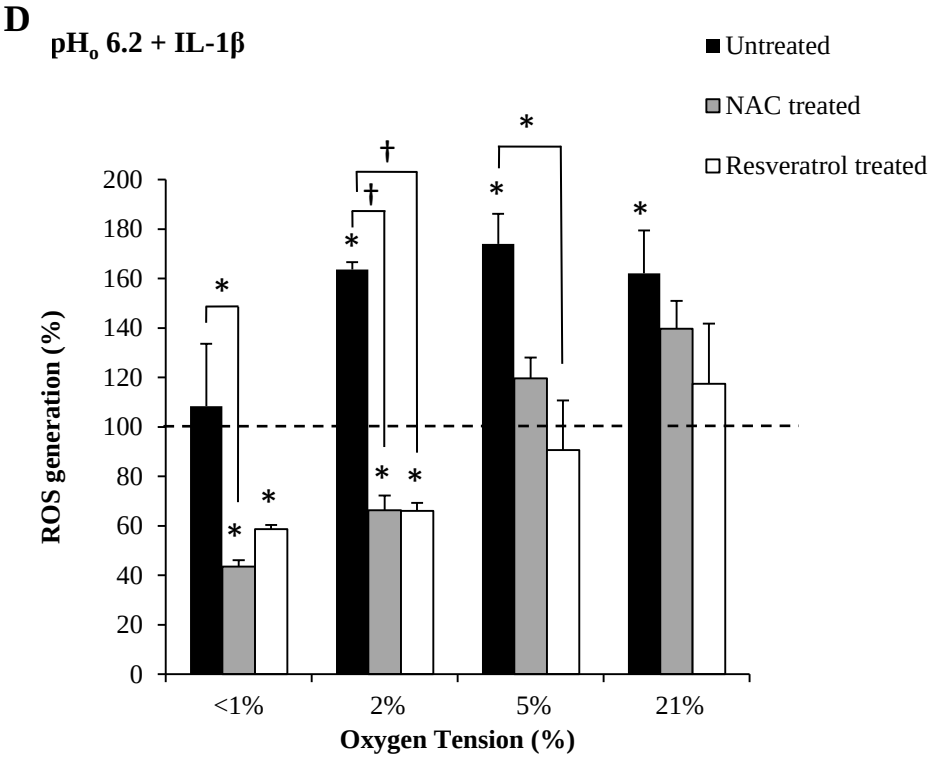


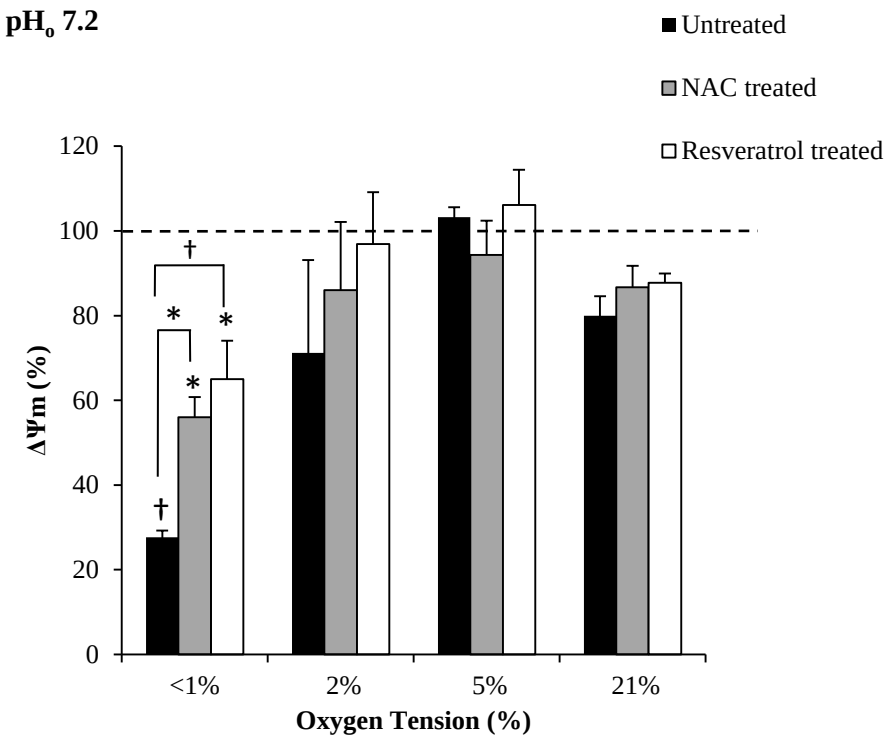
Figure 5.4. Effect of oxygen, pH, IL-1β and antioxidant compounds on EQHAC ROS generation. ROS generation was determined by the DCF method and presented as mean values compared to control (time=0, 5%O_2, pH7.2). EQHAC were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (**A**), 6.2 (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and DCF fluorescence was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.0101compared to control.

5.4.3. Mitochondrial membrane potential

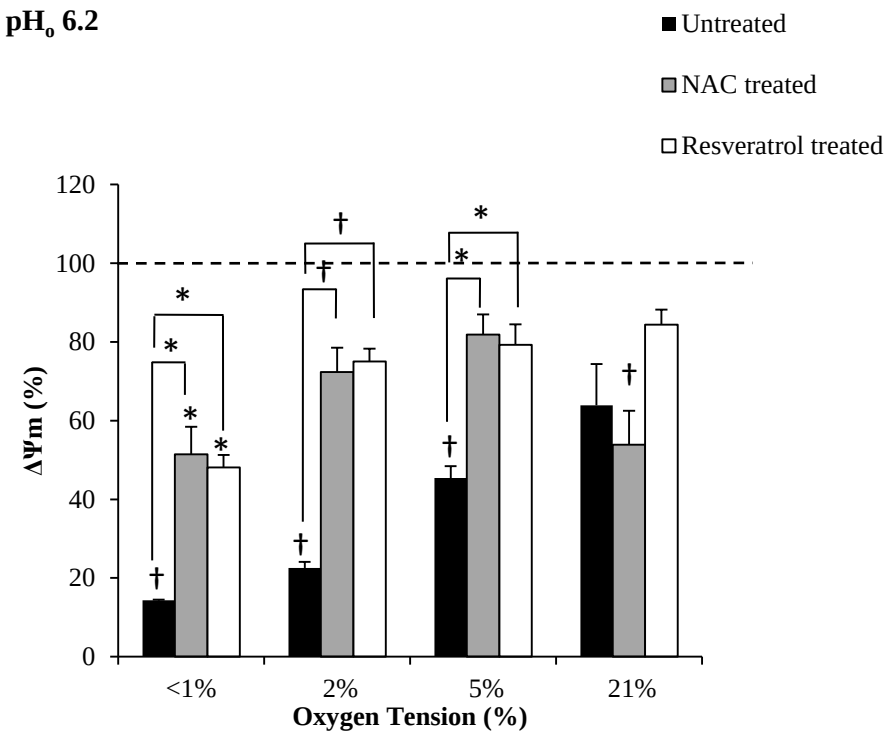
As hypoxia and acidosis markedly altered ROS responses in EQHAC their effects on the ΔΨm of normal equine articular chondrocytes was investigated. Incubation at 2%, 5% or 21% O_2 (pH 7.2) did not significantly alter mitochondrial membrane potentials compared to control values after 48 hours **(**Figure **5.5A**). Culture in anoxia however significantly reduced mitochondrial polarisation to <30% of control (*P*=0.001). Acidosis led to significant mitochondrial depolarisation in O_2 tensions lower than 21%, which appeared oxygen dependent (figure **5.5B**). Thus, as the oxygen tension was reduced from 5% to 2% and <1%, the ΔΨm was reduced from 45.4 ± 3.02% (P=0.001) to 22.5 ± 1.5% (P=0.002) and 14.3 ± 0.18% (*P*=0.001). IL-1β-induced mitochondrial depolarisation was evident in every condition studied and values were reduced by a minimum of 50% compared to control (pH 7.2) (figure **5.5C**). This effect was accentuated in acidic conditions (figure **5.5D**).

Both NAC and resveratrol maintained mitochondrial polarisation to control values in 2%, 5% and 21% O_2 tensions (pH7.2). Incubations with NAC or resveratrol in anoxia however, despite significantly increasing polarisation compared to AOX untreated values, were still significantly lower than control. Again, NAC and resveratrol stabilised the ΔΨm to control values in all acidic conditions except anoxia. Treatment with NAC and resveratrol significantly reduced IL-1β induced mitochondrial depolarisation, partially restoring ΔΨm in both pH 7.2 and pH 6.2 conditions. Results from this part of the study demonstrate that anoxia and acidosis lead to significant reductions in mitochondrial polarisation in normal chondrocytes, an effect partially or totally alleviated by NAC or resveratrol, which worked through mitochondrial membrane potential stabilisation.

A


pHo 7.2
Untreated
NAC treated
Resveratrol treated
120
100
80
60
40
20
0
ΔΨm (%)
<1%
2%
5%
21%
Oxygen Tension (%)

B


pHo 6.2
Untreated
NAC treated
Resveratrol treated
120
100
80
60
40
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0
ΔΨm (%)
<1%
2%
5%
21%
Oxygen Tension (%)

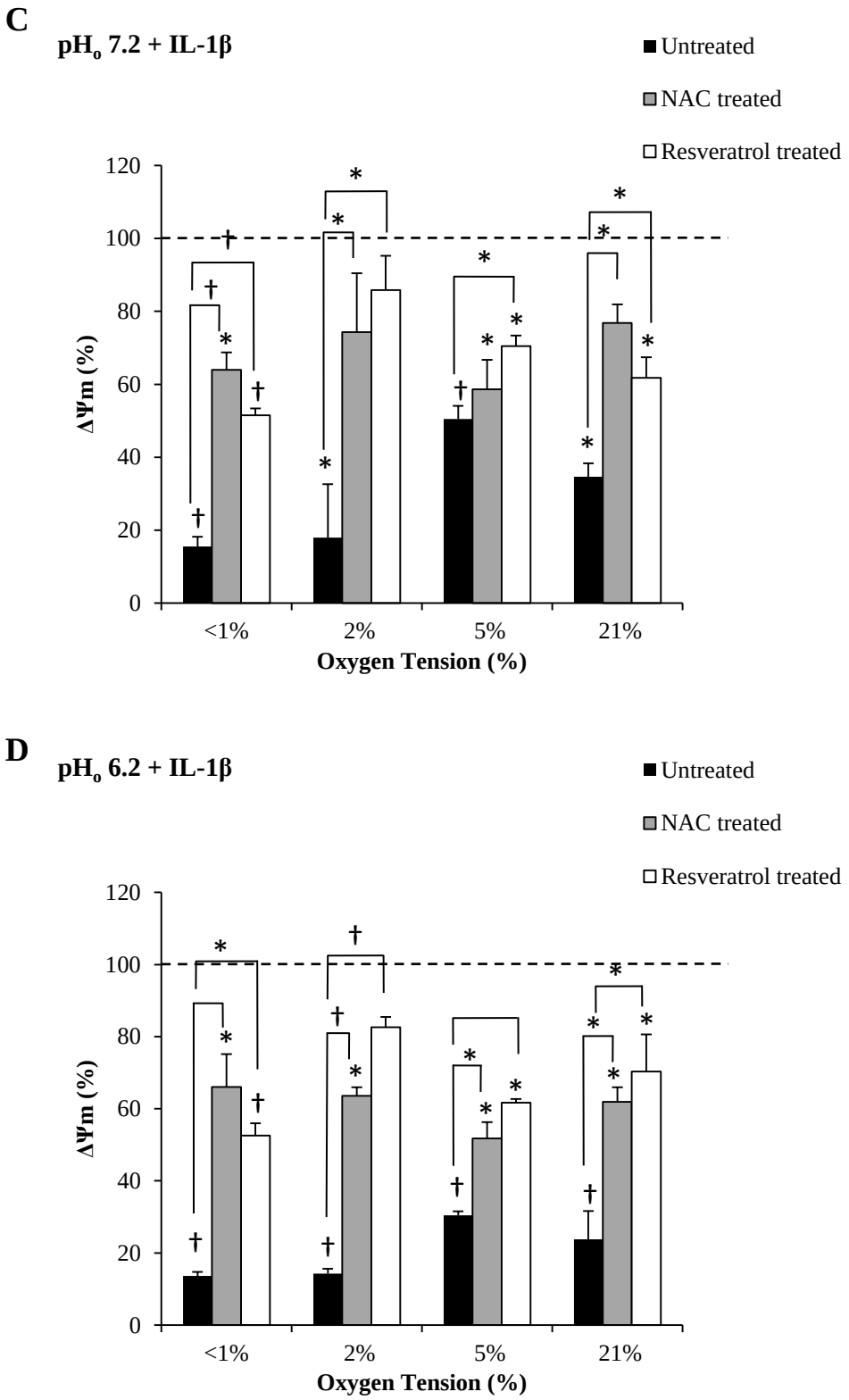


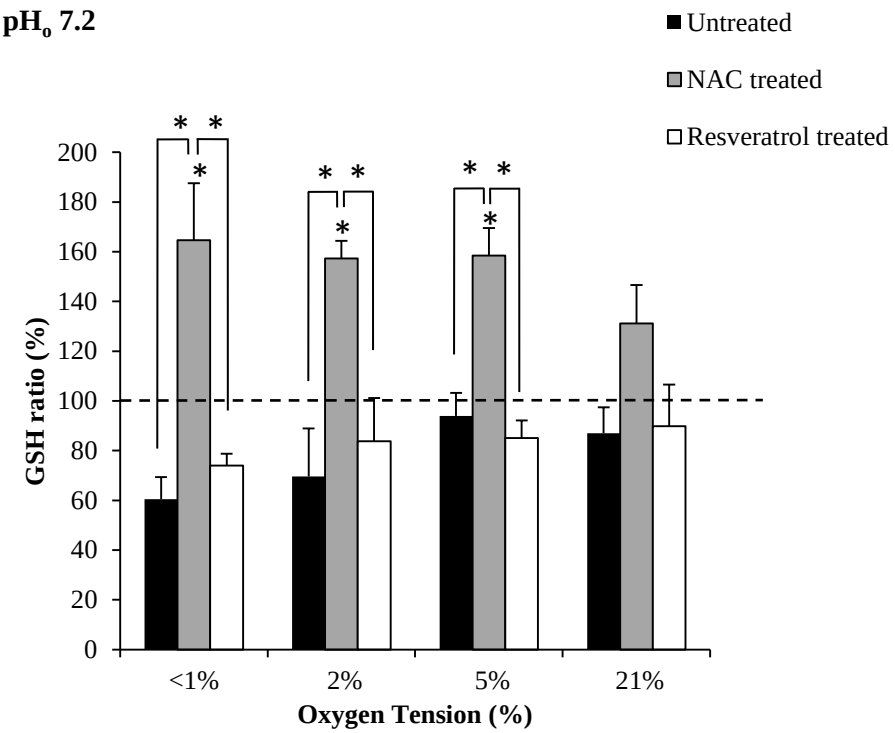
Figure 5.5. Effect of oxygen, pH, IL-1β stimulation and antioxidant compounds on EQHAC ΔΨm after 48 hours. ΔΨm was determined using the fluorescent probe JC-1 and results are presented as mean values compared to control (time=0, 5%O_2, pH7.2). Normal equine chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and ΔΨm was measured at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01compared to control.

5.4.4. GSH:GSSG ratio

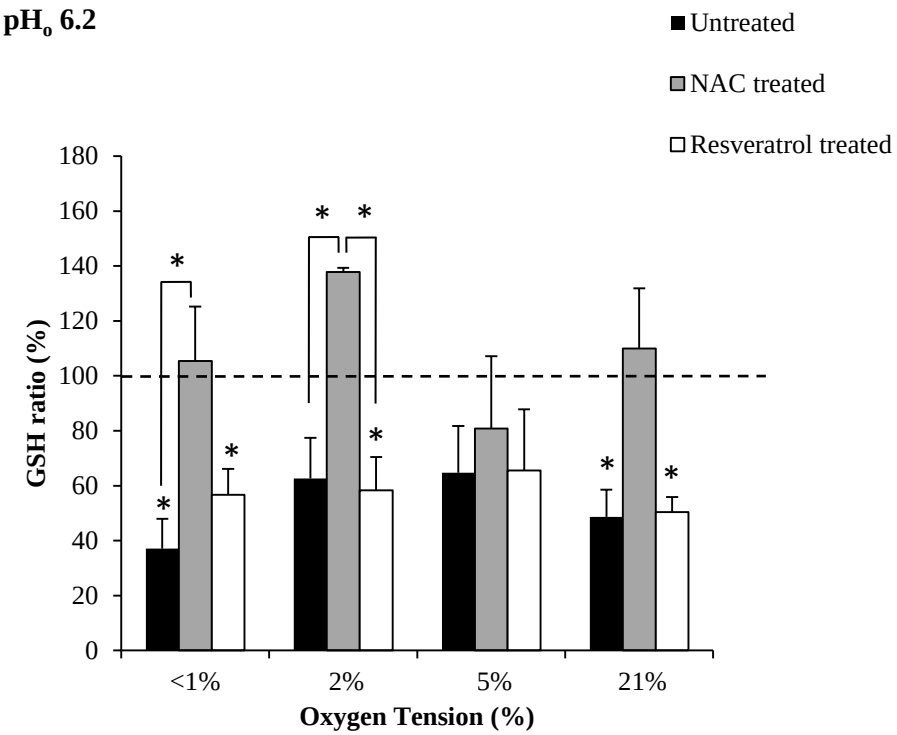
The GSH:GSSG ratio was unmodified by oxygen tension under pH 7.2 conditions after 48 hours (figure **5.6A**). Acidosis significantly reduced the GSH:GSSG ratio in <1% and 21% O_2 tensions compared to control (37.0 ± 10.8% (*P*=0.004), 48.6 ± 9.8%, (*P*=0.004) (figure **5.6B**). The addition of IL-1β (pH 7.2) reduced the GSH:GSSG ratio at physiological pH, reaching significance in <1%, 5% and 21% oxygen conditions (**figure 5.6C**). Acidosis and IL-1β significantly reduced the GSH:GSSG ratio in all O_2 tensions studied, particularly in anoxia were values fell to ~25% of control (*P*=0.001) (figure **5.6D**). No effect of resveratrol addition was evident in this study. Addition of NAC however, powerfully modulated the GSH:GSSG ratio, increasing the ratio to, or above, control values in all O_2 tensions studied. NAC abrogated anoxic and IL-1β-induced reductions of GSH independently of oxygen tension.

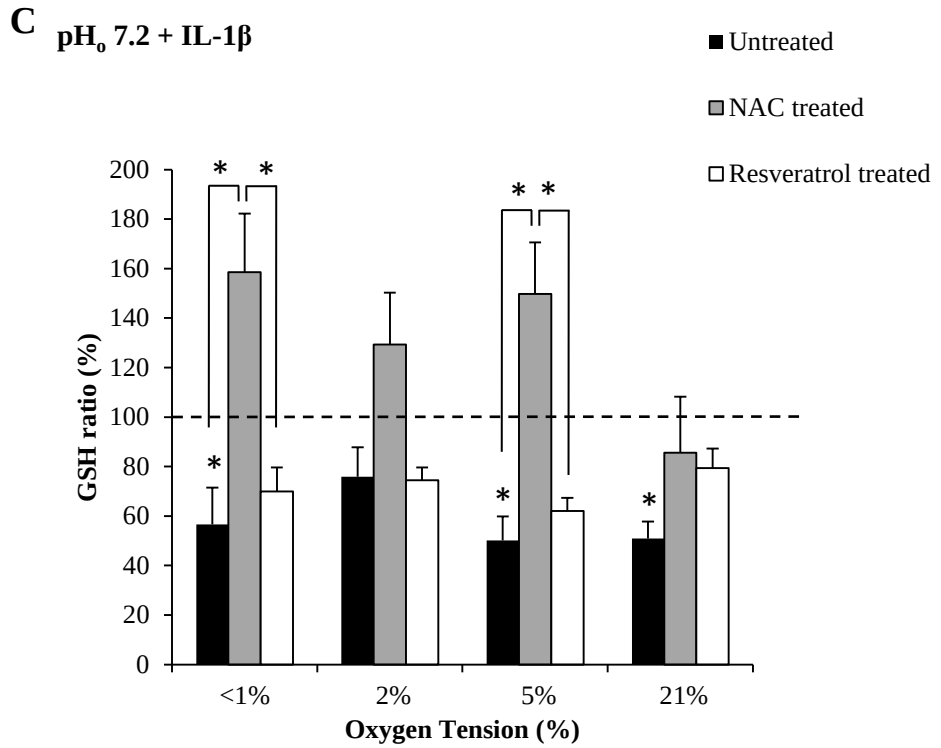
Changes to the GSH:GSSG ratios were a result of fluctuating levels of GSH. GSSG levels remained consistently low in this study and were unaffected by varying oxygen tension, pH, IL-1β or NAC and resveratrol treatment (figure **5.7 A-C**). GSH levels were significantly increased in NAC treated cultures (compared to control) and appeared unaffected by oxygen, pH or IL-1β, peaking at a ratio of 13:1 (in 5% O_2, pH 7.2 conditions).

A **pH_o 7.2**



B **pH_o 6.2**





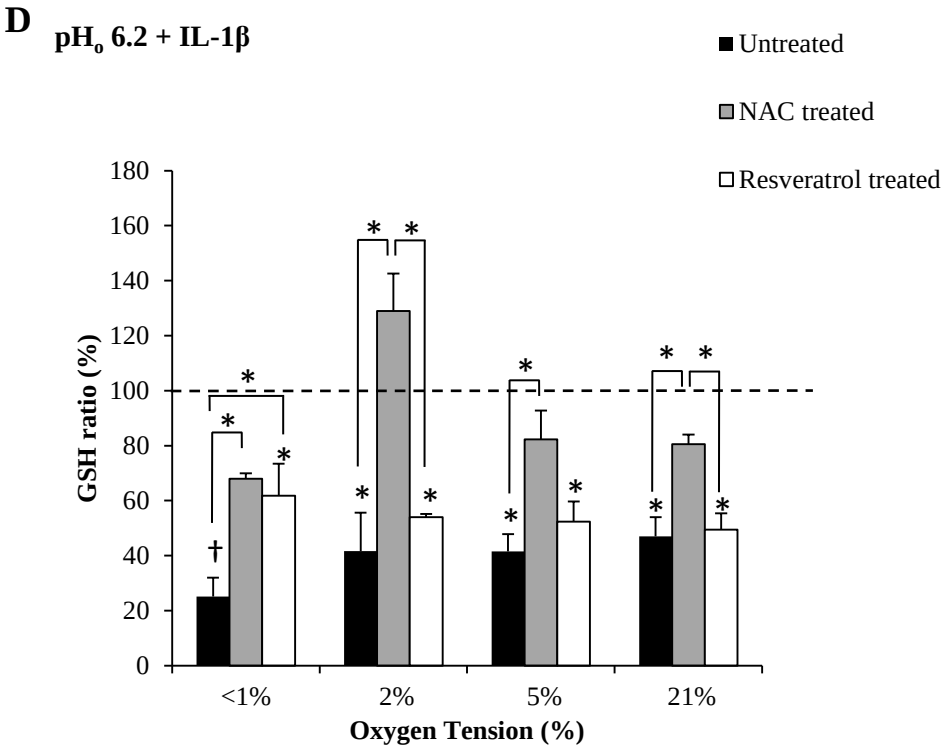


Figure 5.6. Effect of oxygen, pH, IL-1β stimulation and antioxidant compounds on EQHAC GSH:GSSG ratio after 48 hours. GSH:GSSG was measured using the GSH/GSSG-Glo™ Assay and results are presented as mean values compared to control (time=0, 5%O_2, pH7.2). Normal equine chondrocytes were cultured in three dimensional alginate beads in <1%, 2%, 5%, or 21% O_2 at pH 7.2 (normal pH) (**A**), 6.2 (acidosis) (**B**), pH 7.2 plus IL-1β (**C**) or pH 6.2 plus IL-1β (**D**) in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM) and GSH:GSSG ratio was calculated at 48 hours. Bar chart represent means ± SEM, n=3 donors. *P=<0.05; †P=<0.01compared to control.

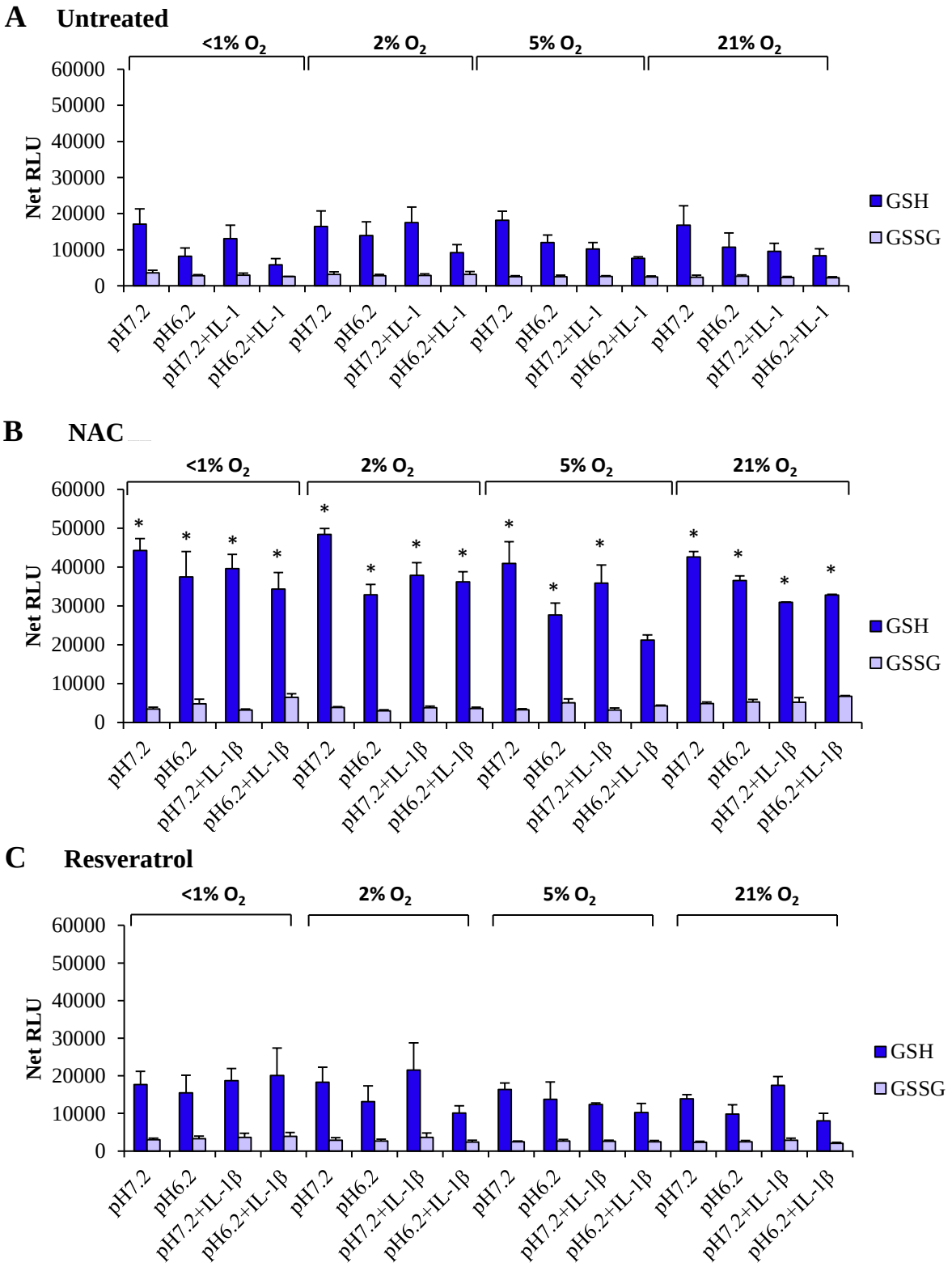


Figure 5.7. Reduced (GSH) and oxidised (GSSG) glutathione levels in normal EQHAC. Panel **A demonstrates** the effect of O_2 tension, pH and IL-1β on GSH and GSSG levels in normal equine articular chondrocytes. Panels **B** and **C** demonstrate the effect of NAC and resveratrol on GSH and GSSG values in normal equine articular chondrocytes respectively. Bar charts represent means ± SEM, n=3donors. Asterisks (*) indicate significant differences compared to control, P=<0.05 compared to control.

5.4.5. SOD1 and SOD2 protein expression in non-diseased EQHAC

Due to the observed effect of acidosis and NAC treatment on the GSH:GSSG ratio, their effects on SOD1 and SOD2 protein expression in non-diseased equine chondrocytes was investigated. Band densitometry analysis reveals a significant effect of anoxia to reduce SOD1 protein expression, an effect abrogated by NAC and resveratrol addition.

Figure **5.8** shows representative full lane control (t=0) (**A**) and experimental (t=48 hours) (**B**) western blots showing SOD1 and SOD2 protein expression. Similarly, figure **5.9** shows representative protein expression of normal equine chondrocytes exposed to <1%, 2%, 5% or 21% O_2, in normal or acidic pH, in the presence or absence of IL-1β, NAC or resveratrol. Band densitometry was performed on all western blots (n=3) and data is presented in table **5.2** (in the absence of IL-1β) and table **5.3** (in the presence if IL-1β).

SOD1 protein expression was unchanged from control values in oxygen tensions above <1%, regardless of culture in acidosis or exposure to IL-1β. Culture in anoxia however, significantly reduced SOD1 protein expression to <60% of control in IL-1β-free conditions and to <30% of control values in the presence of IL-1β. NAC and resveratrol abolished anoxia-induced inhibition of SOD1 protein expression and increased values above control (particularly NAC), although values were non-significant. The expression of SOD2, despite being reduced in normal and acidic pH in anoxia, remained insignificant (table **5.2**). No other effect of oxygen, pH or NAC and resveratrol were evident in pH 7.2 and pH 6.2 conditions in this study. When IL-1β was added to cell cultures, SOD2 was significantly reduced, regardless of pH (table **5.3**). Again, the addition of NAC or resveratrol abolished the combined inhibitory effect of anoxia plus IL-1β on SOD2 and maintained protein expression to control values.

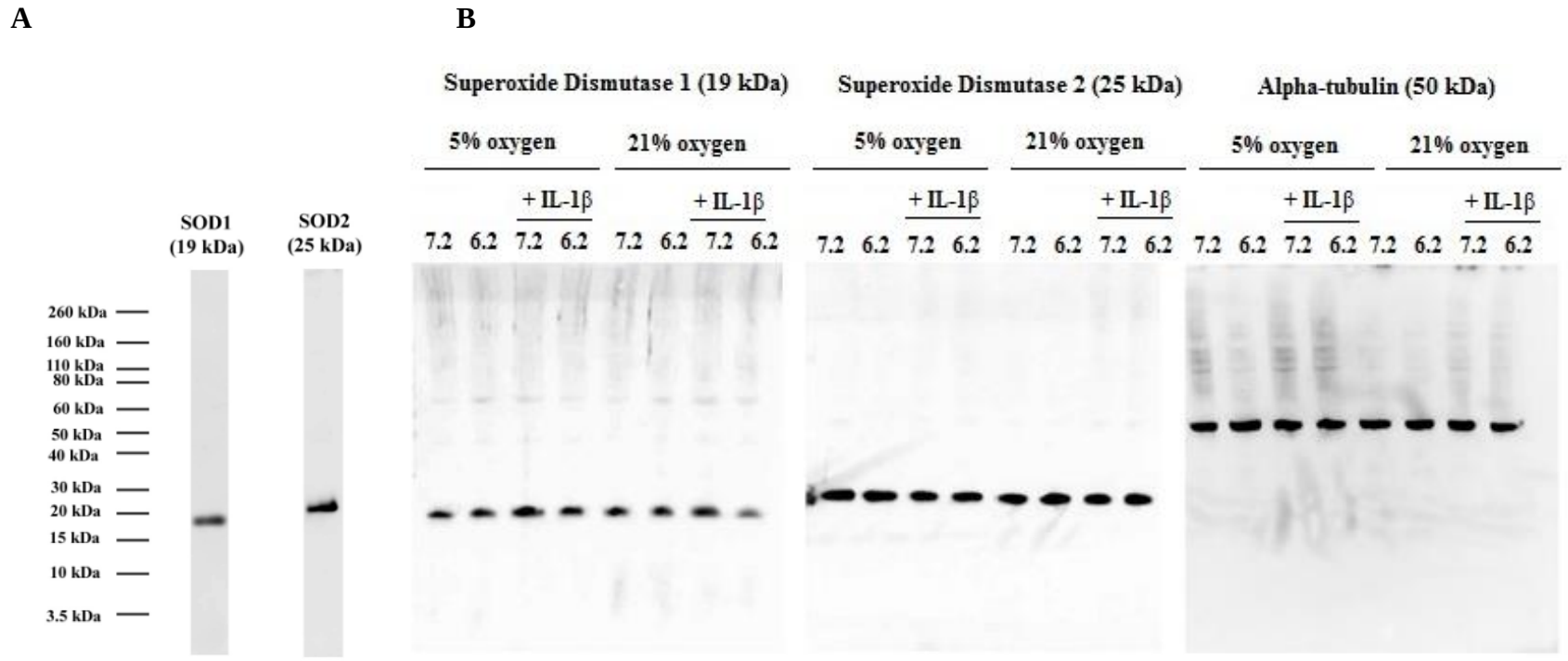


Figure 5.8. Representative full lane blots showing control SOD1 and 2 expression (t=0) (A) and experimental SOD1 and 2 expression and α-tubulin bands (t=48) from one normal equine specimen (B). Lanes 1-8 shows representative western blots of superoxide dismutase expression in EQHAC exposed to 5 and 21% oxygen tension in the absence (pH 7.2) and presence of acidosis (pH 6.2) and IL-1β.

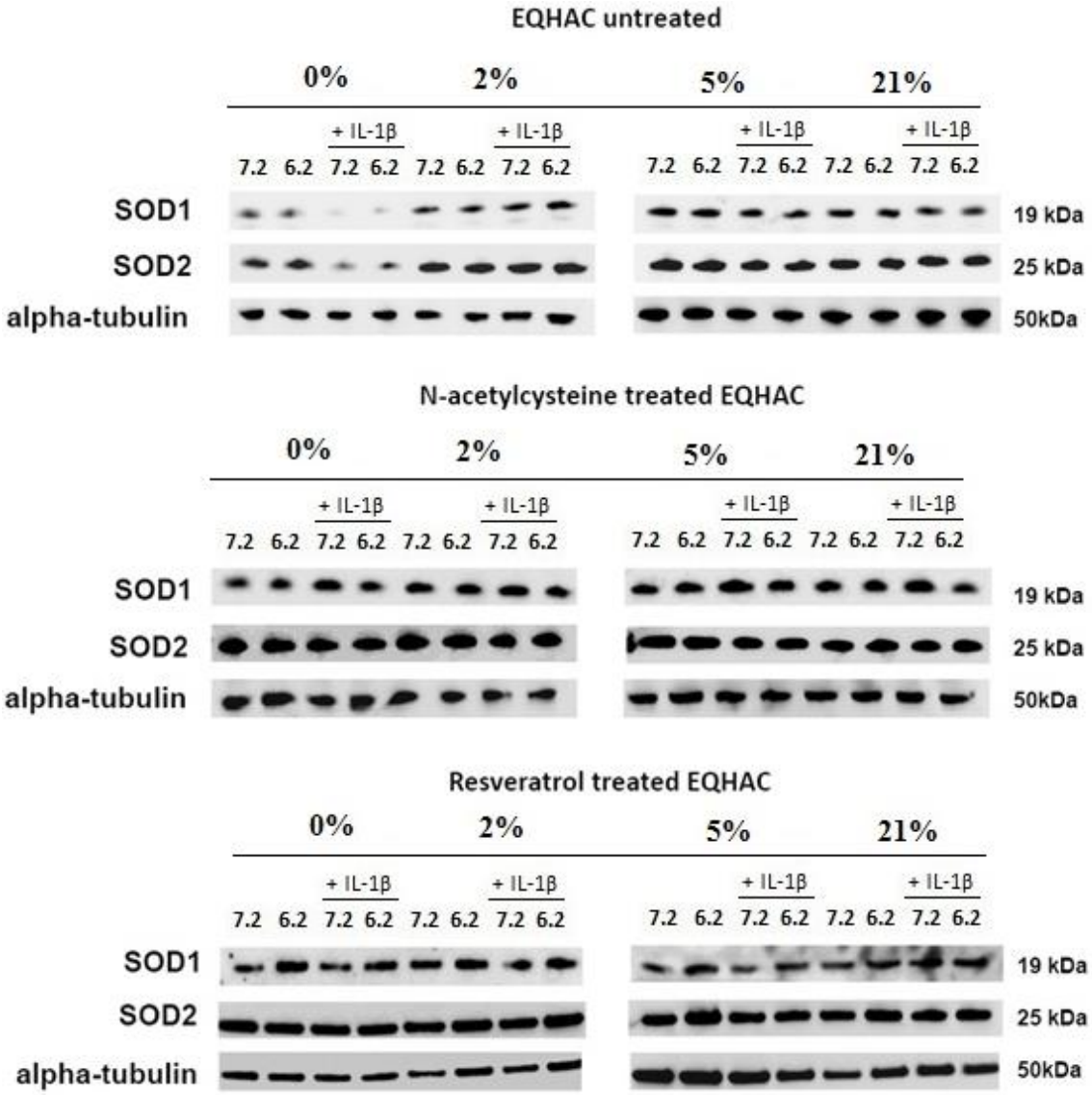


Figure 5.9. Representative western blots showing SOD1 and 2 protein expression of EQHAC cultured in varying O_2 tensions, pH and IL-1β in the absence or presence of N-acetylcysteine (2mM) or resveratrol (10µM). HOAC (n=3) were cultured in 3-D alginate beads in <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the absence or presence of IL-1β (10ng/ml), NAC (2mM) or resveratrol (10µM) (n=3 donors). After experimental incubation cells were released from alginate encapsulation, lysed, separated via SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and incubated in primary antibodies against SOD1 and SOD 2. Alpha-tubulin was used as a loading control.

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
Control	**5%**	**7.2**	100.0 ± 4.03	-	100.1 ± 2.09	-
UNTREATED	**<1%**	**7.2**	59.7 ± 2.8	**0.032 ***	50.4± 21.1	0.124
	2%		94.3 ± 21.6	1.00	118.6 ± 19.2	0.114
	5%		96.6 ± 15.4	1.00	126.2 ± 5.6	0.599
	21%		94.1 ± 16.1	0.97	124.0 ± 5.6	0.582
	<1%	**6.2**	51.1 ± 11.0	**0.011***	51.0 ± 18.3	0.150
	2%		92.5 ± 18.1	1.00	97.0 ± 22.4	0.567
	5%		83.5 ± 15.6	0.81	113.4 ± 8.0	0.534
	21%		78.9 ± 11.6	0.39	124.0 ± 10.3	0.316

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
NAC TREATED	**<1%**	**7.2**	110.3 ± 22.8	0.992	126.4 ± 13.6	0.837
	2%		168.5 ± 26.7	0.434	126.2 ± 12.9	0.624
	5%		187.6 ± 49.6	0.222	146.1 ± 17.9	0.056
	21%		184.2 ± 50.22	0.262	135.5 ± 1.4	0.126
	<1%	**6.2**	124.5 ± 31.8	0.857	155.9 ± 10.5	0.435
	2%		212.0 ± 61.0	0.109	144.9 ± 24.3	0.214
	5%		167.6 ± 49.6	0.414	156.8 ± 2.9	**0.019***
	21%		144.9 ± 26.5	0.746	137.9 ± 13.7	0.098

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
RESVERATROL TREATED	**<1%**	**7.2**	124.4 ± 5.9	0.783	127.6 ± 40.3	0.957
	2%		160 ± 40.1	0.803	125.8 ± 30.3	0.988
	5%		143.3 ± 19.3	0.616	125.5 ± 19.2	0.405
	21%		110.4 ± 25.5	0.997	82.5 ± 10.5	0.537
	<1%	**6.2**	134.0 ± 21.3	0.559	105.7 ± 24.8	1.000
	2%		155.3 ± 48.5	0.843	134.7 ± 68.4	0.966
	5%		130.1 ± 19.3	0.835	120.6 ± 4.7	0.574
	21%		144.6 ± 49.2	0.638	103.0 ± 9.3	0.998

Table 5.2. Quantified SOD1 and SOD2 protein expression of normal equine chondrocytes. Band densitometry was used to calculate protein expression in lysates of EQHAC exposed to <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the absence or presence of NAC (2mM) or resveratrol (10µM) (n=3 donors). Values were normalised to loading control and expressed as percentages compared to control values. Table represents means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
Control	**5%**	**7.2**	100.0 ± 4.03	-	100.1 ± 2.09	-
UNTREATED	**<1%**	**7.2+IL-1β**	29.8 ± 12.8	**0.002***	43.2 ± 28.0	**0.036***
	2%		103.9 ± 25.6	1.00	103.3 ± 18.9	1.000
	5%		74.4 ± 14.9	0.53	115.2 ± 9.8	0.376
	21%		81.6 ± 1.3	0.50	118.4 ± 5.3	0.274
	<1%	**6.2+IL-1β**	23.0 ± 5.3	**0.002***	38.7 ± 7.0	**0.024***
	2%		104.4 ± 21.0	1.00	121.6 ±	0.781
	5%		80.5 ± 14.4	0.72	121.0 ± 3.2	0.136
	21%		70.7 ± 5.2	0.17	91.7 ± 8.9	0.818

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
NAC TREATED	**<1%**	**7.2+IL-1β**	115.9 ± 22.6	0.968	108.7 ± 39.4	0.996
	2%		229.5 ± 53.5	0.061	140.3 ± 10.0	0.287
	5%		152.9 ± 10.6	0.608	126.6 ± 13.0	0.352
	21%		159.2 ± 31.3	0.536	144.1 ± 14.7	0.050
	<1%	**6.2+IL-1β**	115.9 ± 20.5	0.964	113.9 ± 30.1	0.435
	2%		206.1 ± 46.7	0.136	148.8 ± 20.3	0.165
	5%		147.7 ± 31.3	0.683	161.7 ± 10.8	**0.046***
	21%		125.9 ± 26.5	0.944	143.0 ± 12.8	0.057

Treatment	O_2	pH_o	SOD1	P-value	SOD2	P-value
RESVERATROL TREATED	**<1%**	**7.2+IL-1β**	134.2 ± 18.4	0.553	141.1 ±	0.852
	2%		173.1 ± 69.0	0.689	145.9 ±	0.916
	5%		128.9 ± 21.3	0.853	100.6 ±	1.000
	21%		104.7 ± 18.8	1	87.6 ± 8.0	0.771
	<1%	**6.2+IL-1β**	108.5 ± 30.4	0.993	108.6 ±	0.999
	2%		176.6 ± 57.0	0.657	112.5 ±	0.999
	5%		150.9 ± 46.7	0.491	104.5 ±	0.996
	21%		156.1 ± 20.0	0.461	113.2 ±	0.748

Table 5.3. Quantified SOD1 and SOD2 protein expression of normal equine chondrocytes in the presence of IL-1β. Band densitometry was used to calculate protein expression in lysates of EQHAC exposed to <1%, 2%, 5%, or 21% O_2 for 48 hours at pH 7.2 or 6.2 in the presence of IL-1β (10ng/ml), NAC (2mM) or resveratrol (10µM) (n=3 donors). Values were normalised to loading control and expressed as percentages compared to control values. Table represents means ± SEM, n=3 donors. *P=<0.05; †P=<0.01 compared to control.

5.5. Discussion

Chapters 3 and 4 demonstrated the powerful effects of oxygen and pH on cell function and redox balance in human osteoarthritic chondrocytes. However, to determine whether these effects were tissue-specific or as a result of disease (osteoarthritis) it was necessary to examine non-diseased tissue. Normal or mildly affected human articular cartilage is often unavailable and therefore cartilage from comparative species is commonly used. Animal models studying chondrocyte physiology generally involve murine, lapine, canine, bovine and equine tissues, with cartilage thickness and joint size generally corresponding to animal size (Chu et al, 2010). The metacarpophalangeal joint of skeletally mature horses, with no signs of joint damage were used in this investigation. The horse offers several advantages in the absence of normal human cartilage. Horses suffer from cartilage arthropathies and disorders such as osteochondritis dissecans, fibrillations, erosions and OA, similar to the human. Indeed, the field of equine cartilage pharmaceuticals is well advanced (Brommer et al, 2003; Chu et al, 2010). Compared to other animals, horses provide a close approximation in terms of cartilage thickness to humans.

This chapter analyses the distinct and interactive effects of oxygen tension and pH, as well as exposure to IL-1β, NAC and resveratrol, on normal equine articular chondrocyte redox function and mitochondrial homeostasis, using the *in vitro* model detailed in chapter 3. By using normal articular cartilage with no signs of OA, the effects of physiological as well as pathophysiological levels of oxygen and pH could be evaluated, potentially mimicking global changes within the joint in non-diseased joints and in early disease (for example in inflammatory synovitis, where IL-1 levels are also increased).

Oxygen-sensitivity of normal equine chondrocyte mitochondria and redox status

Similar to results in Chapters 3 and 4, culture of non-diseased equine articular chondrocytes in a 5% O_2 environment led to the least deviations from control values in this study which is consistent with previous literature on normal chondrocytes, from a variety of species, which estimate this oxygen tension as normoxic (Fermor et

al, 2007; Henrotin et al, 2005; Lafont, 2010; Murphy & Polack, 2004; Silver & Maroudas, 1975; Zhou et al, 2004). Additionally, chondrocyte phenotype was unaffected by oxygen tension. Oxygen consumption remained steady state in higher oxygen tensions (21% O_2), which is in contrast to findings from the previous chapter in OA chondrocytes, and may suggest an ability of normal chondrocytes to maintain normal metabolic homeostasis in the face of increasing oxygen environments. Cell survival, ROS levels, mitochondrial polarisation and SOD1 protein expression however, all displayed marked oxygen sensitivity after 48 hours in this study. Reductions in oxygen tension from 5% to <1% O_2 led to reductions in these parameters. Indeed anoxic culture was significantly damaging.

No differences in ROS levels or mitochondrial polarisation were apparent when normal equine chondrocytes where cultured in 5% and 21% O_2 tensions after 48 hours in this study. These observations corroborate the findings from chapter 4 and extend them, showing that ROS levels are inhibited by reduced oxygen tension (down to <1%) after 48 hours culture. Anoxia induced reductions in ROS levels correlated with mitochondrial depolarisation which is consistent with the hypothesis that mitochondrial ROS production in physiological conditions is influenced by mitochondrial electron flux through the ETC. Thus it appears that the modulatory effect of oxygen tension on ROS levels and $\Delta\Psi m$ is not species specific and suggests that equine cartilage may be a suitable tissue to analyse redox and mitochondrial pathways, with relation to OA pathology. Oxygen mediated mitochondrial dysfunction, such that is demonstrated here, likely explains the reduced chondrocyte survival observed in this study. Prolonged hypoxia has been shown to reduce chondrocyte mitochondrial function (Ruiz-Romero et al, 2009) and apoptotic cues are usually preceded by mitochondrial alterations. Reductions in mitochondrial polarisation are an early response associated with programmed cell death in many tissues (Petit et al, 1996; Zamzami et al, 1996) including chondrocytes (Dave et al, 2008). Indeed, mitochondrial depolarisation is associated with altered mitochondrial function, release of cytochrome *c* from the mitochondrial ETC and subsequent activation of caspases which initiate pro-apoptotic machinery (Granville and Gottlieb, 2002). In the present study, anoxia significantly reduced cell viability which correlated with increased mitochondrial depolarisation. This could suggest

that modulation and stabilisation of ΔΨm may inhibit cellular apoptosis and protect the chondrocyte from oxygen mediated stress, providing a possible target for therapeutic intervention.

This study also demonstrates a significant effect of anoxia to inhibit SOD1 protein expression. This finding is in agreement with others that demonstrate oxygen sensitivity of SOD in chondrocytes (Ruiz-Romero et al, 2009) and other tissues. Adachi and coworkers for example demonstrate that low oxygen tension reduces SOD activity and increases pro-apoptotic caspase release in retinal pericytes (Adachi et al, 2011). IL-1β reduced SOD1 protein expression further in anoxia, but had no effect on SOD expression in higher oxygen tensions suggesting that in our study, effects of IL-1β display oxygen sensitivity. IL-1β also led to increases in ROS levels, indicative of the chondrocyte respiratory burst mechanism, highlighting the powerful effect of this pro-inflammatory cytokine on chondrocyte behaviour. Increased ROS release was significantly elevated in low and high oxygen tensions and suggests a regulatory effect of oxygen on IL-1β efficacy.

Of note in this study was the ability of normal equine chondrocytes to withstand a hypoxic (exposure to 2% O_2) challenge. Mitochondrial polarisation and GSH:GSSG ratios remained at control values despite culture in hypoxia (GSH:GSSG ratios were also maintained despite anoxic treatment). This is in contrast to observations in OA chondrocytes (chapter 4) were hypoxia stimulated significant mitochondrial depolarisation and reductions in the GSH:GSSG ratio. Thus it appears that normal chondrocytes are well equipped to maintain redox homeostasis and mitochondrial function during mid-term hypoxic culture through maintenance of antioxidant systems. Furthermore, an increased susceptibility to hypoxic stress is evident in OA chondrocytes, which perturbs redox and mitochondrial homeostasis and compromises cellular integrity. Addition of IL-1β is heavily implicated in OA pathology and compromises chondrocyte integrity. Exposure of normal chondrocytes to IL-1β massively inhibited GSH:GSSG content and significantly depolarised chondrocyte mitochondria, the latter being oxygen dependent, with lower ΔΨm being observed as the oxygen tension is reduced.

Alterations in redox status and mitochondrial function by extracellular pH

Culture in acidosis did not alter COL2A1 or SOX9 expression after 48 hours, demonstrating the ability of normal equine cells to maintain a chondrocyte phenotype over this time course (which is in contrast to the findings in OA chondrocytes). Similarly, GAG release and cell viability appeared unmodified by acidosis (even in the face of hypoxia and/or IL-1β), which suggests normal chondrocytes are well equipped to handle prolonged environmental stress *in vitro*. This study however does show that acidosis is an important regulator of ROS generation, mitochondrial polarisation and GSH:GSSG ratios in normal equine chondrocytes, which presumably would affect cellular ECM components, cellular phenotype and survival if maintained.

Exposure to acidosis led to increases in ROS generation, marked mitochondrial depolarisation and reductions in GSH:GSSG ratios in normal equine chondrocytes which is heavily suggestive of oxidative stress. These results are similar to those observed in OA chondrocytes (Chapter 4). ΔΨm and GSH:GSSG ratios also showed oxygen sensitivity (being further reduced as oxygen tensions are lowered), highlighting the powerful dual role of these environmental parameters on redox balance and mitochondrial homeostasis in normal chondrocytes. A combination of low oxygen tension and acidosis are clinical features of OA and therefore these conditions aimed to mimic a diseased environment. The dis-coordinated responses observed and concomitant oxidative stress would likely manifest into reduced survival and matrix integrity.

The effects of NAC and resveratrol to modulate redox balance and mitochondrial function in normal equine chondrocytes

By inducing mitochondrial dysfunction and redox imbalance in normal chondrocytes and testing the ability of ROS-regulatory compounds to restore normal mitochondrial and redox function, we are likely to further increase our understanding of OA pathology and uncover therapeutic targets for management of OA. As such, we tested the ability of NAC and resveratrol to modulate ROS levels, mitochondrial

polarisation, GSH:GSSG ratios and SOD1/2 expression in normal chondrocytes exposed to a range of oxygen tensions, acidosis and IL-1β.

Antioxidant abilities of NAC

Treatment with NAC negated IL-1β induced ROS overproduction in higher oxygen tensions (5 and 21% O_2) but had no effect on normal chondrocyte ROS production when levels were lowered (2% and <1% O_2). NAC also partially restored the ΔΨm of normal equine chondrocytes and fully restored anoxic and IL-1β induced SOD inhibition back to basal levels. The latter finding is in agreement with others who demonstrate that SOD levels are oxygen sensitive and are reduced by hypoxia in rat pericytes. Addition of NAC to pericyte cultures abolished this effect and increased SOD levels to control values which correlated with reduced markers of apoptosis (caspase-3) and positive modulation of hypoxia related genes (VEGF) (Adachi et al, 2011). The role of NAC in regulating cell survival in chondrocytes is well documented and has also been shown to positively affect proteoglycan content and to confer protection against injury from blunt impact which may be particularly important when considering changes to normal loading patterns associated with early OA and its progression (Martin et al, 2009).

The greatest effect of NAC in this study however, was on the GSH:GSSG ratio. Treatment with NAC led to abrogation of acidosis and IL-1β-induced reductions in GSH. Treatment with NAC significantly increased GSH levels in every condition tested (independent of acidosis or IL-1β), and with no change in GSSG being observed, led to significant increases in the GSH:GSSG ratio of normal articular chondrocytes. These findings are in agreement with our previous results in OA chondrocytes, but demonstrate an increased ability of NAC to heighten GSH levels (all significant). This effect again is most likely through increasing the cysteine pool and subsequent GSH synthesis (as described in chapter 4). These findings highlight the antioxidant potential of GSH supplementation on environmentally induced GSH depleted normal chondrocytes (which may be an early event in OA pathology) and highlights a potential therapeutic target.

Antioxidant abilities of resveratrol

Findings from this chapter demonstrate the antioxidant abilities of resveratrol to maintain ROS levels, to partially or totally restore mitochondrial polarisation and to positively modulate SOD expression. These results agree with and extend the findings from the previous chapter from OA chondrocytes. Perhaps one of the major findings of this study was the ability of resveratrol to maintain ROS levels despite anoxic or acidotic culture. Addition of resveratrol abolished the anoxic-inhibition and acidosis-induced increase of ROS observed in resveratrol untreated conditions. This finding is especially important in light of recent findings that demonstrate ROS as key cellular signalling messengers necessary for appropriate chondrocyte function. During hypoxia, ROS levels are reduced (as is observed in this study), which leads to perturbations of ionic homeostasis and intracellular acidification (Milner et al, 2006). Restoration of ROS through exogenous addition of H_2O_2 or pharmacological manipulation of the mitochondrial ETC recovers intracellular acidification (Milner et al, 2007). Work in this chapter demonstrates that resveratrol exerts the same ROS-restoring ability. It is likely that partial or total maintenance of mitochondrial polarisation in the presence of resveratrol (in all oxygen tensions) explains this finding and implicates appropriate mitochondrial function as a key determinant of redox homeostasis in chondrocytes, which is consistent with others (Cillero-Pastor et al, 2013; Blanco et al, 2011). Treatment with resveratrol also reduced anoxia-induced LDH release which may have occurred due to resveratrol-mediated stabilisation of mitochondrial polarisation.

The role that ROS plays as a secondary messengers has intensified and it is now accepted that ROS signalling is an important step in activation/inactivation of many pathways such as those governed by calcium and lipids, G protein, protein tyrosine kinase and protein tyrosine phosphatase (Kamata and Hirata, 1999). The modulatory effect of anoxia on ROS levels, as shown in this study, implicates the surrounding oxygen environment as a key regulator of ROS generation and therefore ROS signalling. In turn, the effect of resveratrol to abolish oxygen mediated ROS inhibition through modulation of mitochondrial function demonstrates the potential of this compound to contribute to such pathways. Further work to detail these effects

is warranted and may lead to therapeutic strategies in diseases where mitochondrial dysfunction and redox imbalance are known features (such as OA).

Resveratrol also negated the effect of anoxic and IL-1β induced SOD inhibition which further demonstrates the antioxidant potential of resveratrol in maintaining redox balance in articular chondrocytes. This observation is in accordance with others who demonstrate the ability of resveratrol to reduce pharmacologically induced oxidative stress by increasing antioxidant expression, particularly SOD levels (Spanier et al, 2009). The effects of resveratrol as a direct antioxidant are rather poor and at the µM concentration used in the present study (10 µM), the ability of resveratrol to directly scavenge ROS has been shown to be as low as 2.8% (Hung et al, 2002). Rather, the present study suggests that the powerful antioxidant potential of resveratrol is through manipulation of mitochondrial membrane potentials and SOD expression. Through mitochondrial stabilisation, resveratrol abolishes anoxia-induced ROS inhibition, returning ROS generation to basal levels and also prevents ROS overproduction through maintenance of SOD.

Resveratrol also displays a close relationship with NRF2 (Ungvari et al, 2004) and alongside the work detailed in chapter 4 (showing hypoxic perturbations of NRF2 expression), points to a potential therapeutic pathway, although it was beyond the scope of this study to explore it and represents a potential avenue of future research.

5.6. Conclusion.

To conclude, this chapter investigated the distinct and interactive effects of different oxygen tension and extracellular pH on redox homeostasis and mitochondrial function of normal equine articular chondrocytes following 48 hours incubation. The ability of antioxidant compounds NAC and resveratrol to alter oxygen and acidosis mediated changes to redox and mitochondrial homeostasis were also analysed. ROS generation, mitochondrial polarisation and SOD1 protein expression all displayed significant oxygen sensitivity, being reduced in anoxia. Normal articular chondrocytes also displayed pH sensitivity with acidosis significantly altering ROS levels, reducing $\Delta\Psi m$ and significantly inhibiting GSH content. Exposure to anoxia and acidosis was especially detrimental to normal chondrocyte function and amplified responses, causing marked oxidative stress. Addition of NAC and resveratrol preserved oxygen and acidosis-induced redox imbalance through distinct mechanisms. Treatment with NAC significantly increased GSH content of chondrocytes and abrogated acidosis and IL-1β induced reductions in GSH:GSSG ratios. Treatment with resveratrol restored anoxia-induced ROS inhibition through restoration of $\Delta\Psi m$ which may be particularly relevant in redox signalling and led to increased cell survival. Finally, both NAC and resveratrol exerted antioxidant abilities by abrogating anoxic and IL-1β-induced inhibition of SOD levels, maintaining expression to values comparable to control.

Chapter 6

Summary

6.1. Introduction: Environmental challenges to chondrocytes in health and disease

The chondrocyte is the only resident cell in articular cartilage and as such is responsible for the maintenance of the ECM and therefore the integrity of cartilage as a whole (Lafont, 2010). Crucially, if lost, chondrocytes are not replaced, leaving the remaining cells with an increased burden for the maintenance of the ECM. Perturbations in chondrocyte function ultimately lead to dis-coordinated responses, altered matrix homeostasis and joint diseases such as OA (Schneider et al, 2003). Chondrocytes respond and adapt to changes in the local cellular environment and thus, alterations in this environment significantly modulate cellular function. Due to the avascular nature and the functional demand of articular cartilage, this tissue is typified by fluctuating levels of hypoxia and acidosis compared with other cells. Fluctuations in these environmental parameters are cues that chondrocytes respond to, the levels of which are altered under normal (due to the dynamic nature of the tissue) and diseased conditions (Gibson et al, 2008). The responses of normal and OA chondrocytes to changes in oxygen tension and extracellular pH form the basis of this project. This study specifically focuses on the role of oxygen tension and extracellular pH to modify redox balance and mitochondrial function in chondrocytes. Appropriate redox balance and mitochondrial homeostasis is vital for cellular function but the effects of the local environment on redox and mitochondrial pathways in chondrocytes have gone unexplored, despite being altered in OA. Consequently, this study tested the hypothesis that fluctuations in oxygen tension and extracellular pH would lead to alterations in redox balance and mitochondrial function, ultimately affecting chondrocyte homeostasis.

6.2. Effects of O_2 tension and pH on chondrocyte redox balance and mitochondrial function

This study shows that the oxygen tension in which chondrocytes are cultured is a key determinant of cellular function (tables **6.1** and **6.2**). An oxygen tension of 5% was beneficial for articular chondrocyte homeostasis and led to the least deviation from control values in all parameters analysed, consistent with the work of others.

Lowering oxygen levels from 5% (to hypoxic or anoxic levels) led to reductions in ROS generation, SOD protein expression, NRF2:KEAP1 ratios, ATP_i levels and caused intracellular acidification, mitochondrial depolarisation and activation of HIF-1α in articular chondrocytes. Many of these observations are potentially linked. For instance, mitochondrial depolarisation likely explains the reduction in ROS due to reduced electron flux in the mitochondrial ETC. Similarly, the observed inhibition of ATP_i is likely an effect of reduced substrate availability (negative Pasteur effect) (Lee and Urban, 1997). Increasing the oxygen tension to ambient levels (21%) was also damaging to articular chondrocytes and led to enhanced nitric oxide production and GAG release and increased chondrocyte oxygen consumption, the latter suggestive of altered metabolism and a switch towards oxidative phosphorylation. Extracellular acidosis was also a crucial determinant of chondrocyte behaviour. Reducing the pH of extracellular media from physiological values (pH 7.2) to acidic values commonly recorded in diseased cartilage (pH 6.2) led to reductions in cell viability, GSH: GSSG ratios and induced mitochondrial depolarisation. Extracellular acidosis also increased ROS levels and GAG release and inhibited ATP_i levels.

Perhaps the major finding of this study however was the combined effects of low oxygen tension and acidosis on articular chondrocyte function (summarised in **figure 6.1**). Oxygen tensions and extracellular pH are lowered from normal values during joint diseases (Levick et al, 1990; Zhou et al, 2004) yet this is the first study to our knowledge that investigates the combined effects of low oxygen tension and extracellular pH on chondrocyte function *in vitro*. Hypoxia/anoxia and acidosis led to significant increases in ROS levels and also caused massive mitochondrial depolarisation and reductions in SOD expression and GSH:GSSG ratios. These results are highly suggestive of perturbed mitochondrial homeostasis and a propensity towards oxidative stress, the effects of which affected downstream parameters such as GAG release (increased), cell viability (reduced) and chondrocyte phenotype (reduction in COL2A1 and SOX9 expression). It is highly likely that the observed increase in ROS levels in these conditions is related to the reductions in ROS regulatory enzymes. The mechanism behind the paradoxical increase in ROS levels despite a reduction in mitochondrial membrane potential remains unidentified and highlights that further work in this area is required.

	HOAC				EQHAC			
O_2	**<1%**				**<1%**			
pH	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**
IL-1β	-	-	+	+	-	-	+	+
COL2A1 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
SOX9 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
GAG release	↔	↑	↑	↑	↔	↔	↔	↔
LDH release	↔	↔	↔	↑	↑	↑	↑	↑
ROS generation	↓	↓	↔	↔	↓	↔	↔	↔
Mitochondrial polarisation	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
GSH/GSSG ratio	↓↓	↓↓	↓↓	↓↓	↔	↓	↓	↓↓
SOD1 expression	↔	↔	↔	↔	↓	↓	↓	↓
SOD2 expression	↔	↔	↔	↔	↔	↔	↓	↓

	HOAC				EQHAC			
O_2	**2%**				**2%**			
pH	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**
IL-1β	-	-	+	+	-	-	+	+
COL2A1 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
SOX9 expression	↔	↔	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
GAG release	↔	↔	↔	↑	↔	↔	↔	↑
LDH release	↔	↔	↔	↔	↔	↔	↔	↔
ROS generation	↔	↔	↔	↔	↔	↔	↑	↑
Mitochondrial polarisation	↓↓	↓↓	↓↓	↓↓	↔	↓↓	↓↓	↓↓
GSH/GSSG ratio	↓↓	↓↓	↓↓	↓↓	↔	↔	↔	↓
SOD1 expression	↔	↔	↔	↔	↔	↔	↔	↔
SOD2 expression	↔	↔	↔	↔	↔	↔	↔	↔

Table 6.1.Summary table demonstrating the effects of low O_2 levels (<1%, 2% O_2), pH_o (7.2 and 6.2) and IL-1β on AOX untreated HOAC and EQHAC in this study. Horizontal arrows indicate no change in parameter tested. Single arrows indicate significant differences where P=<0.05. Double arrows indicate significant differences where P=<0.01 versus control (time-0, 5% O_2, pH 7.2).

	HOAC				EQHAC			
O_2	**5%**				**5%**			
pH	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**
IL-1β	-	-	+	+	-	-	+	+
COL2A1 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
SOX9 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
GAG release	↔	↔	↔	↑	↔	↔	↔	↔
LDH release	↔	↔	↔	↑	↔	↔	↔	↔
ROS generation	↔	↔	↑	↑	↔	↔	↔	↑
Mitochondrial polarisation	↔	↓↓	↓	↓↓	↔	↓↓	↓↓	↓↓
GSH/GSSG ratio	↔	↓↓	↓↓	↓↓	↔	↔	↓	↓
SOD1 expression	↔	↔	↔	↔	↔	↔	↔	↔
SOD2 expression	↔	↔	↔	↔	↔	↔	↔	↔

	HOAC				EQHAC			
	21%				**5%**			
pH	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**	**7.2**	**6.2**
IL-1β	-	-	+	+	-	-	+	+
COL2A1 expression	↔	↓	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
SOX9 expression	↔	↔	**N/A**	**N/A**	↔	↔	**N/A**	**N/A**
GAG release	↔	↔	↔	↑	↔	↔	↔	↔
LDH release	↔	↔	↔	↔	↔	↔	↔	↔
ROS generation	↔	↑	↑	↑	↔	↔	↑	↑
Mitochondrial polarisation	↔	↓↓	↓↓	↓↓	↔	↔	↓	↓↓
GSH/GSSG ratio	↔	↓↓	↓↓	↓↓	↔	↓	↓	↓
SOD1 expression	↔	↔	↔	↔	↔	↔	↔	↔
SOD2 expression	↔	↔	↔	↔	↔	↔	↔	↔

Table 6.2.Summary table demonstrating the effects of normoxic and hyperoxic O_2 levels (5%, 21% O_2), pH_o (7.2 and 6.2) and IL-1β on AOX untreated HOAC and EQHAC in this study. Horizontal arrows indicate no change in parameter tested. Single arrows indicate significant differences where P=<0.05. Double arrows indicate significant differences where P=<0.01 versus control (time-0, 5% O_2, pH 7.2).

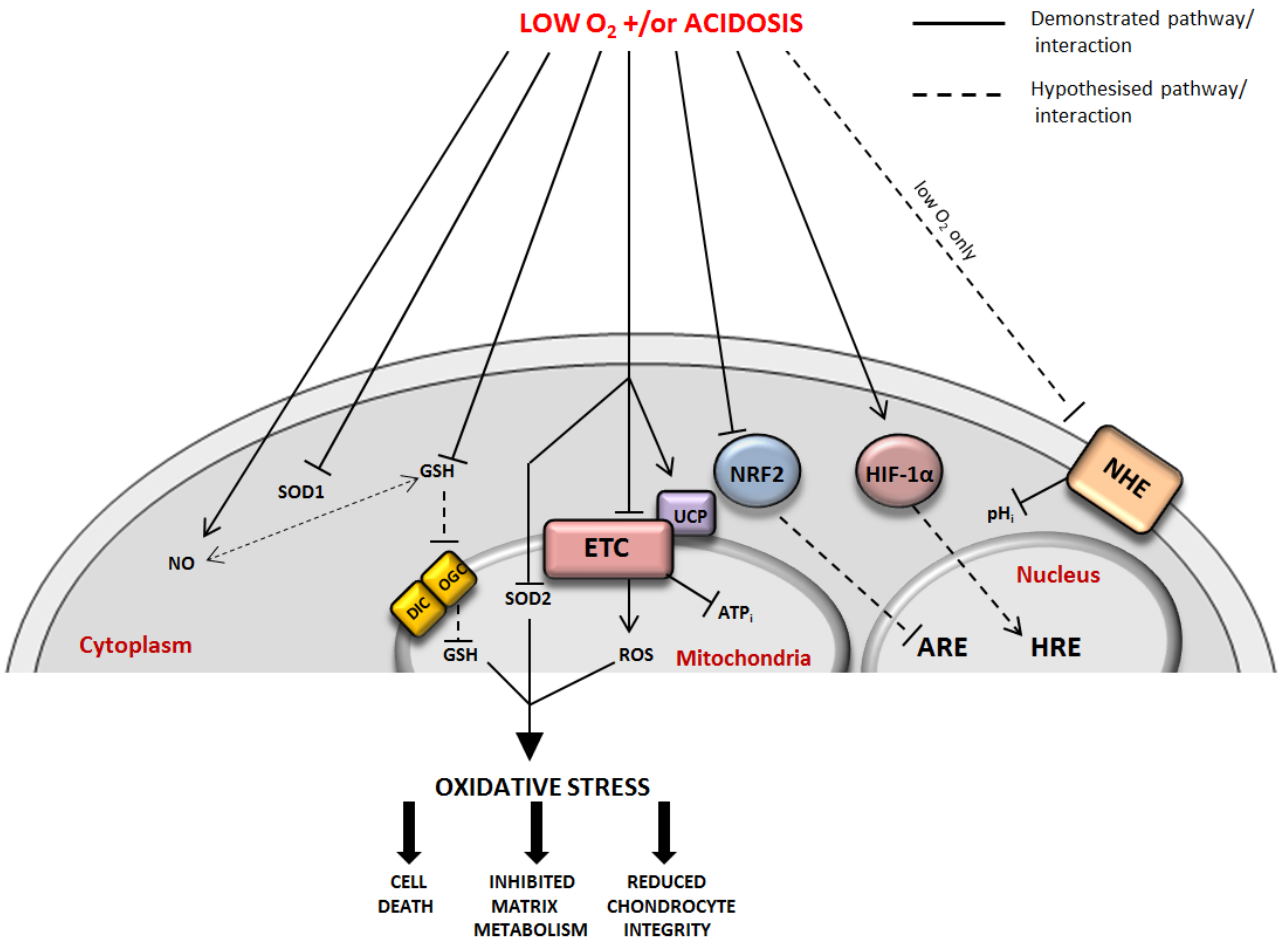


Figure 6.1. The combined effects of low oxygen tension and acidosis on chondrocyte function. Demonstrated and hypothesised interactions found in this study are shown.

6.3. The effects of IL-1β stimulation on redox balance in articular chondrocytes

Upregulation of the pro-inflammatory cytokine, IL-1β in joint disease is a hallmark event. As a result the sensitivity of the activity of IL-1β to oxygen and pH was investigated and its effects on redox and mitochondrial homeostasis under these conditions. This study observed differences in sensitivity to IL-1β between normal equine chondrocytes and OA human chondrocytes. Responses from non-diseased articular chondrocyte were highly manipulated by IL-1β whereas responses in diseased human cartilage where more subtle. Differences are likely to be disease specific, that is to say that chondrocytes from diseased joints are likely to respond differently than non-diseased chondrocytes but species specific differences could also exist. Interestingly, in OA chondrocytes, the effects of IL-1β appeared dependent on pH. The effects of IL-1β were abrogated in acidosis and this cytokine had no significant effect on the variables measured. This is in contrast to physiological pH conditions where addition of IL-1β accentuated observed responses (i.e. further reduced cell viability, GSH: GSSG ratios and increased GAG release and ROS levels). This finding may be particularly important in a clinical setting in light of the reduced pH of the OA joint and the negligible effect of cytokine inhibitor treatment in OA patients (Chevalier et al, 2005; Chevalier et al, 2009; Yang et al 2008).

6.4. Manipulation of redox balance through ROS regulatory compounds

Exogenous addition of compounds that exert ROS-regulatory properties is a growing area of research in the field of OA therapy. This study investigated the effects of two well documented ROS regulatory compounds, N-acetylcysteine and resveratrol. This study suggests that NAC and resveratrol exert distinct antioxidant properties in the face of oxygen or acid-mediated stress. In human OA articular chondrocytes, NAC significantly increased GSH levels and partially maintained mitochondrial polarisation whereas resveratrol markedly reduced ROS levels, increased SOD2 protein expression and maintained $\Delta\Psi m$. In equine chondrocytes from non-diseased joints, similar findings were observed but responses were accentuated. For example, NAC markedly increased the GSH content of chondrocytes and abrogated acidosis

and IL-1β induced reductions in GSH:GSSG ratios. Resveratrol abrogated acidosis and IL-1β induced ROS overproduction and increased anoxia induced ROS inhibition through modifications of ΔΨm. Finally, both NAC and resveratrol exerted antioxidant abilities by abrogating anoxic and IL-1β-induced inhibition of SOD levels thereby alleviating the environmentally induced oxidative stress noted in AOX-free conditions.

6.5. Future directions and concluding remarks

Unlike many studies, this project has focused on the *combined* effects of a number of extracellular environmental parameters associated with disease in how they modify chondrocyte redox balance and mitochondrial function. An increased understanding of the role that environmental parameters exert on chondrocyte function is needed and may lead to novel therapeutics and enhance the fundamental understanding of the pathogenesis of joint disease, such as osteoarthritis. The work detailed in this thesis demonstrates that redox balance and mitochondrial homeostasis are regulated by oxygen and extracellular pH and it appears that many interactions exist, which can ultimately affect chondrocyte function. Further delineating these interactions and targeting molecular pathways to restore or maintain redox balance and mitochondrial function could provide alternative treatment options for a multifactorial disease devoid of symptom-alleviating therapy.

This study is the first to report a modulatory effect of oxygen on the NRF2 pathway in OA chondrocytes. Further elucidating the modulatory effect of environmental parameters on NRF2 and KEAP1 as well as potential interactions with the mitochondrial tethering protein PGAM5 is warranted in light of the highly regulatory role these pathways play in antioxidant and redox responses in other tissues. Activation of HIF-1α in low oxygen environments was also demonstrated. Although this study only briefly considers the role of HIF's on chondrocyte function, the role of these transcription factors are widely accepted as governing many, if not all chondrocyte responses to hypoxia (Pfander et al, 2005; Schipani et al, 2001). Further identifying the potential interactions between HIF and ROS is warranted in light of

recent observations (Archer et al, 2007; Bell et al, 2007), and may give a further insight to the role of ROS in cellular (hypoxic) signalling.

With current treatment for OA only being palliative, supplementation of compounds with postulated ROS-regulatory actions, to alter or prevent disease processes, is a controversial and highly researched area of medical biology (Atkuri et al, 2007). For instance, supplementation with ascorbic acid, a widely accepted antioxidant, has received widespread attention in the field of OA therapy, with many studies demonstrating the ROS regulatory capacity of this compound (Yudoh et al, 2005). Conversely, the work of Kraus et al (2004) demonstrate that ascorbic acid supplementation actually increases cytokine induced osteophyte formation and increases synovial COMP values (an early marker of joint catabolism) in an animal model of OA, thereby augmenting the disease process. Similar discrepancies have been observed with other postulated antioxidant compounds (e.g. eicosapentaenoic acid, α-tocopherol, zyflamend, trace elements) but despite their hypothesised roles in disease prevention (ROS detoxification, anti-inflammatory properties, chondrocyte growth, suppression of cytokine release), it is concluded that currently there is not enough evidence to fully recommend specific antioxidant supplementation as a treatment for OA, despite several compelling lines of evidence. The need for continued research in this field is, however warranted (see Wang et al, 2004, McAlindon et al, 2005, Rosenbaum et al, 2010 for reviews).

Clearly, the postulated effects of antioxidant compounds in OA therapy are diverse and the mechanisms of action not fully elucidated. Such contrasting and diverse findings highlight the need for continued research in the field of redox-regulation by nutritional supplementation. Here, this study has focused on two well-recognised compounds with reported anti-oxidant activities, N-acetyl cysteine and resveratrol and demonstrate distinct ROS-regulatory properties of these compounds to positively modulate redox and mitochondrial homeostasis in articular chondrocyte. This study demonstrates the ability of these molecules to alter environmentally induced stress associated with OA, but the efficacy of treatment with antioxidant compounds in joint disease, remains unclear. It does however provide a basis for their use in therapeutics aimed at joint disease modification or alleviation. Since joint diseases

like osteoarthritis affect millions world-wide, the development of compounds targeting alleviation of joint disease symptoms will continue and it is likely that drugs aiming to modify redox balance in cartilage will be a highly probable target for therapeutic intervention.

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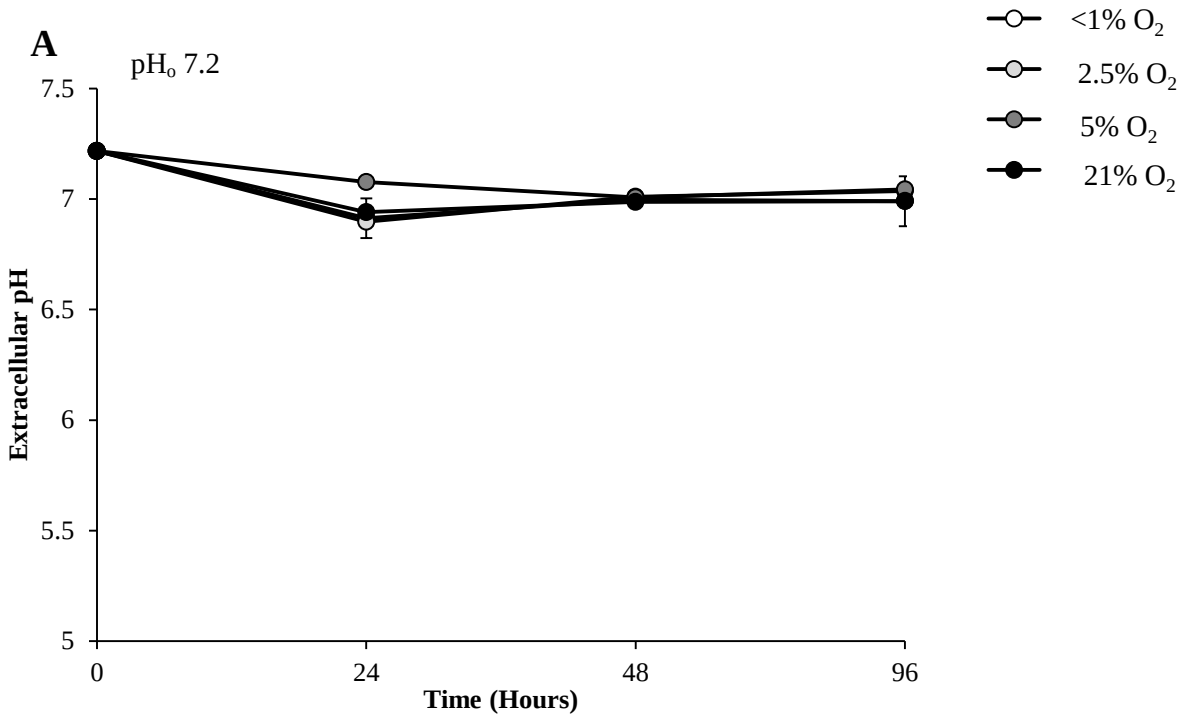
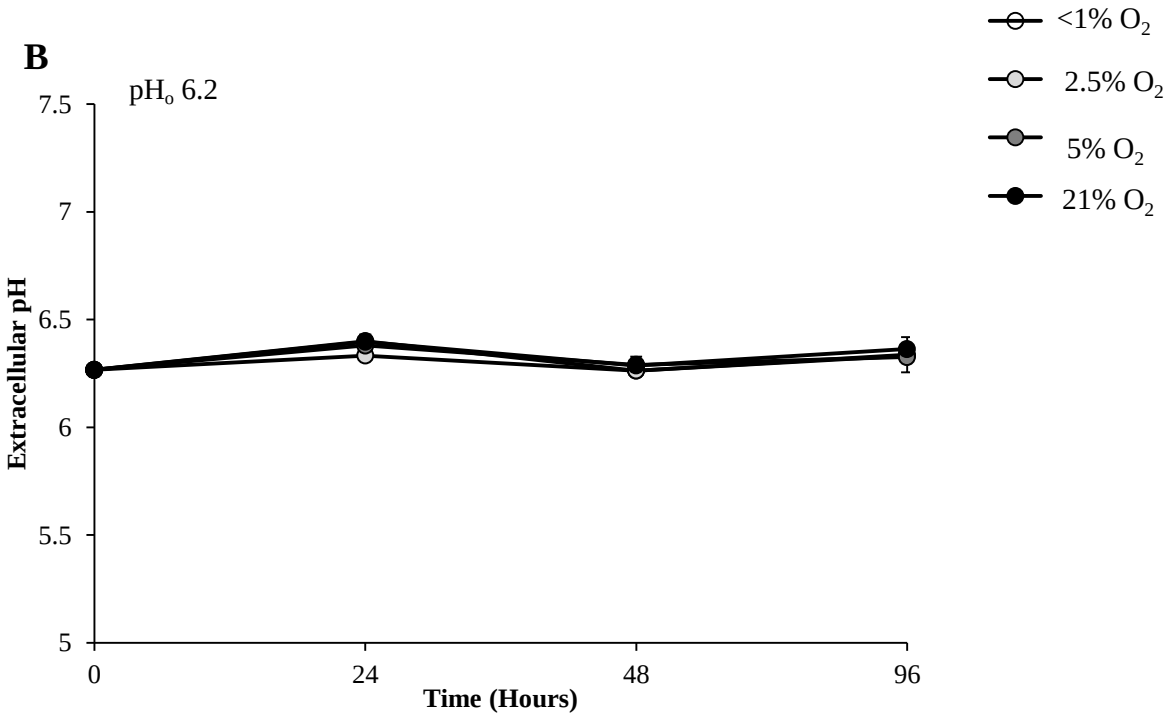
Appendix i

Extracellular pH of cell free media over 96 hours





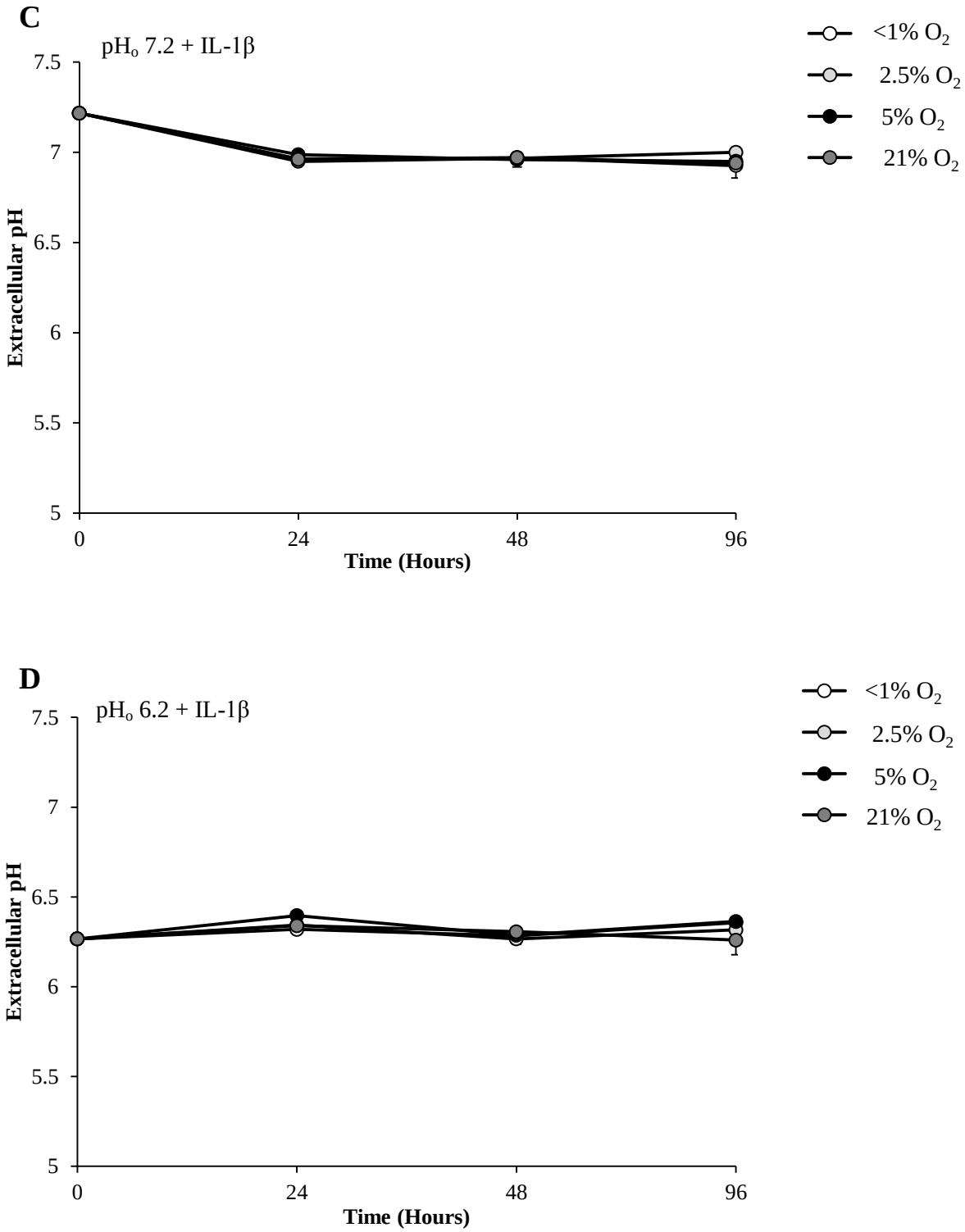


Figure 1. Effect of varying O_2 tension and IL-1β on media pH in the absence of cultured cells. Media was incubated alone for 24, 48 or 96 hours in <1%, 2.5%, 5% or 21% O_2. Panels **A-D** show changes in extracellular pH of normal (pH 7.2) or acidic (pH 6.2) media in the presence or absence of IL-1β. Results represent mean ± SEM pH values (n=3 donors). *$P < 0.05$ compared to control (time-0) (P=<0.05).

Cell number and cell viability of alginate cultured HOAC over 14 days

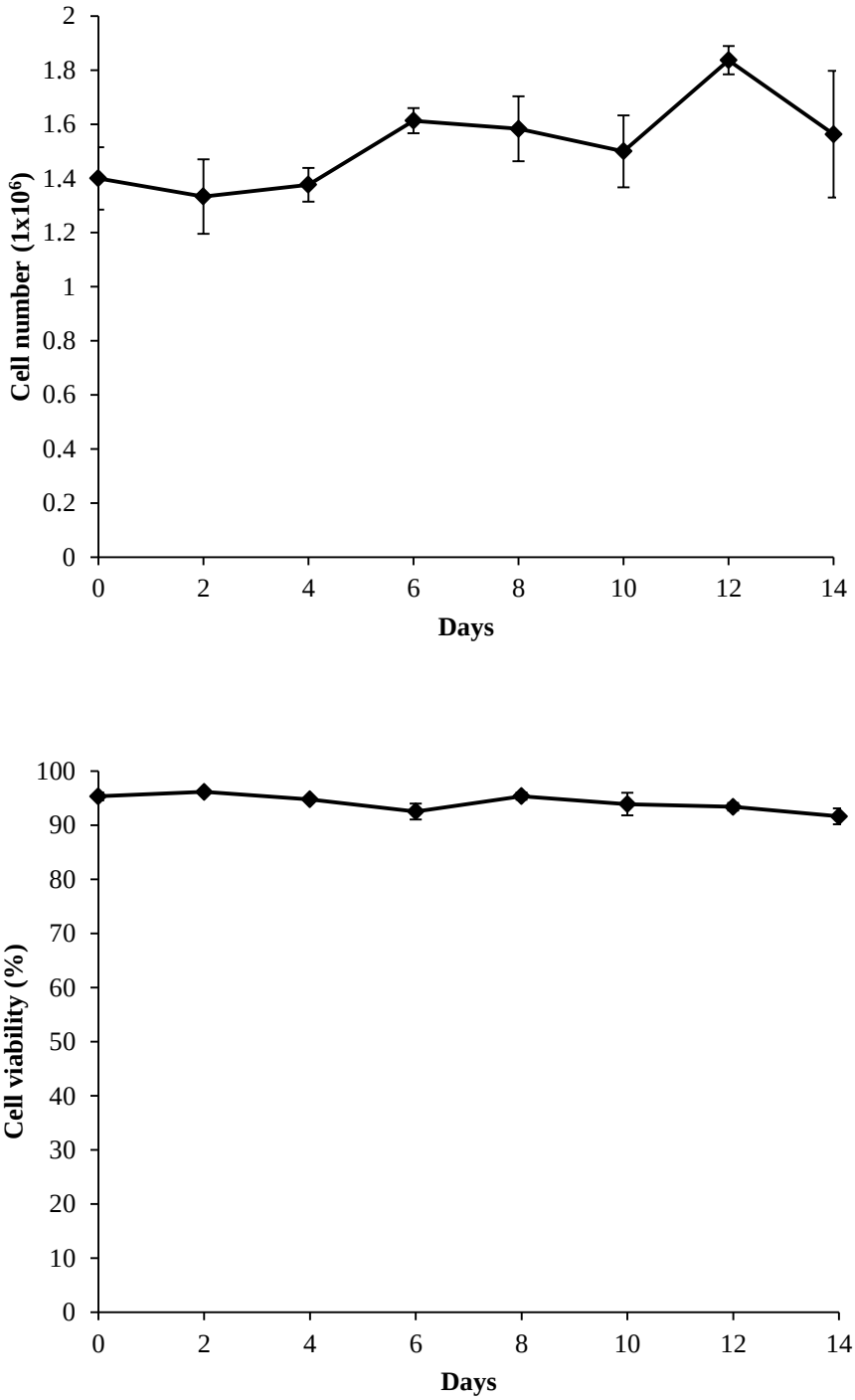


Figure 2. HOAC cell count and viability assessed over 14 days culture in alginate (n=3). Cell viability was determined by Trypan blue exclusion and cell number determined by haemocytometry.

Appendix ii