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NUTRITIONAL TARGETING OF INFLAMMATORY PATHWAYS AND CATABOLIC MEDIATORS INVOLVED IN EQUINE OSTEOARTHRITIS

Abigail Louise Clutterbuck, BSc., MSc.

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

May 2011

Declaration

I declare that this thesis, and the data presented in it are my own work. I have generated most of the information presented as a result of my own original research except where otherwise stated. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what work was done by all parties involved.

Chapter 2, section 2.6, study 4. This study was conducted with the help of Timothy Rogers as part of his undergraduate dissertation. I designed the study, helped with the plate set-up, and performed the assays and data analysis. Mr Rogers collected the explants, set up the plates, collected and froze the explants and media after 5 days.

In chapter 4, a cocktail of plant extracts was developed by Dr Pat Harris, Claire Barfoot and Isabel Harker at MARS Horsecare UK Ltd for use in the model and for the field trial in chapter 5.

In chapter 5, the proteomic processing and some of the data analysis was done by Dr Julia Smith at Bruker Daltonics. I designed and performed the *in vitro* phase of the study and prepared the samples for proteomic analysis, then analysed most of the data and performed the western blots.

In chapter 6, statistical analysis of the field trial data was performed by Alison Colyer from the WALTHAM Centre for Pet Nutrition (WCPN).

Ethical Statement

No horses were sacrificed for the studies in this thesis and all horses from which tissue was obtained were euthanized for purposes other than for research. This was in accordance with the ethical values of the industrial partner (WALTHAM Centre for Pet Nutrition).

Abstract

Osteoarthritis (OA) is a degenerative disease of synovial joints with an inflammatory component, which affects humans and companion animals, including horses. Current pharmacotherapy for OA is associated with deleterious side effects. Therefore, plant-derived products with anti-inflammatory properties may provide safer natural alternatives. The project aimed to use *in vitro* models of equine cartilage to test the hypothesis that plant-derived extracts would reduce inflammation and degradation in an explant model of early OA.

The anti-inflammatory polyphenol, curcumin, significantly reduced interleukin-1beta (IL-1β)-stimulated glycosaminoglycan, matrix metalloproteinase-3 and prostaglandin E_2 release in the explant model. Using a cocktail of plant extracts illustrated how different effects were observed depending on the solvent used to dissolve the raw material. Chondrocyte monolayers were used to determine that counteraction of IL-1β-stimulated effects in the explant model occurred at non-cytotoxic concentrations.

The explant model was adapted for proteomic studies of the cartilage secretome. Several proteins involved in matrix function and degradation were identified. This adaptation may further our understanding of the processes in the early OA explant model and may facilitate studying the effects of anti-inflammatory compounds on the secretome.

A concurrent field trial showed that the plant extract cocktail did not significantly improve mobility in horses with chronic hindlimb stiffness. However, it illustrated the need for practical, more objective markers to help select animals of similar disease status and determine effects in the joints. Therefore, the proteomic study highlighted the potential for *in vitro* models to support field trials by identifying *in vivo* biomarkers for diagnosing early OA and assessing therapeutic responses.

In conclusion, *in vitro* models of equine cartilage have considerable potential for assessing the ability of plant extracts to target inflammatory pathways and catabolic mediators in OA. The data presented suggests that nutritional intervention using plant-derived extracts with putative anti-inflammatory properties may support equine joint health.

Published Papers

Papers Resulting from the Work in this Thesis

Clutterbuck, A.L., Harris, P., Allaway, D. and Mobasheri, A. (2010) **Matrix metalloproteinases in inflammatory pathologies of the horse.** *The Veterinary Journal*, 183(1): 27-38.

Clutterbuck, A.L., Mobasheri, A., Shakibaei, M., Allaway, D. And Harris, P. (2009) **Interleukin-1beta -induced extracellular matrix degradation and glycosaminoglycan release is inhibited by curcumin in an explant model of cartilage inflammation.** *Annals of the New York Academy of Sciences*, 1171: 428–435.

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Graham, N.S., Clutterbuck, A.L., James, N., Lea, R.G., Mobasheri, A., Broadley, M.R. and May, S.T. (2010) **Equine transcriptome quantification, using human GeneChip arrays, can be improved using genomic DNA hybridization and probe-selection**. *The Veterinary Journal*, 186(3): 323-327.

Mobasheri, A., Henrotin, Y., Clutterbuck, A.L., Allaway, D., Lodwig, E.M., Harris, P., Mathy-Hartert, M. and Shakibaei, M. (2010) **Nutritional biochemistry of curcumin (diferuloylmethane) and a review of its biological actions on articular chondrocytes**, in Haugen, S. and Meijer, S. (eds) *Handbook of Nutritional Biochemistry: Genomics, Metabolomics and Food Supply*, Hauppauge, New York: Nova Science Publishers, Inc.

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Mobasheri, A. Csaki, C., Clutterbuck, A.L., Rahmanzadeh, M. and Shakibaei, M. (2009) **Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy.** *Histology and Histopathology* 24: 347-366.

Sutton, S., Clutterbuck, A.L., Harris, P., Gent, T., Freeman, S., Foster, N., Barrett-Jolley, R., Mobasheri, A. (2009) **The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis.** *The Veterinary Journal*, 179(1): 10-24.

Accepted Papers

Accepted Papers Resulting from the Work in this Thesis

Clutterbuck, A.L., Smith, J.R., Allaway, D., Harris, P., Liddell, S. and Mobasheri, A. (2011) **High throughput proteomic analysis of the secretome in an explant model of articular cartilage inflammation**. *Journal of Proteomics*, accepted 15.02.11, available online 24.02.11. doi: 10.1016/j.jprot.2011.02.017.

Accepted Papers Produced During the Course of this Thesis

Mobasheri, A., Asplin, K., Clutterbuck, A.L. and Shakibaei, M. (2010) **Nutraceuticals: From research to legal and regulatory affairs**, in Henrotin, Y. (ed) *Non-pharmacological Therapies in the Management of Osteoarthritis*, U.A.E.: Bentham Science Publishers Ltd.

Acknowledgements

Firstly, I wish to acknowledge the Biotechnology and Biological Sciences Research Council (BBSRC) and the WALTHAM Centre for Pet Nutrition (WCPN), for their generous funding and for enabling me to undertake this project.

I would like to show my appreciation for my supervisors: Dr Ali Mobasheri, Dr Pat Harris and Dr David Allaway for their constant support and advice throughout the course of my project. They have been inspirational leaders and I am indebted to them for their encouragement and guidance through the trials and tribulations of scientific research. Specifically, I would like to thank Ali for his continual motivation; his incredible depth of knowledge never ceases to amaze me. I wish to thank Pat for her sound advice and rational approach to everything; her passion for equine welfare and scientific research is truly inspiring. I also thank David for his support and constructive input, for putting everything into perspective and asking the questions that make you step back and look at the bigger picture. Our meetings were informative, thought provoking and enjoyable. For that, I thank you all.

Many thanks must go to Dr Julia Smith at Bruker Daltonics for her help and support with the proteomics work, as well as Dr Susan Liddell at the School of Biosciences for imparting her vast knowledge and allowing me into her immaculate laboratory.

The technical support provided by Scott Hulme, Dawn Scholey, Morag Hunter, Leigh Sylvester and the effervescent Ken Davies has been invaluable and I am truly grateful to them all for their help and patience.

I also wish to praise Nicky Jarvis and her wonderful team at Redwings Horse Sanctuary for their hospitality and their dedication to the field trial. The whole team of vets, vet nurses, farm managers, checkers and handlers was a pleasure to work

with and their unfailing good humour and warmth made the whole experience thoroughly enjoyable and memorable.

Much gratitude must go to my friends and colleagues at the vet school who have been a great source of help, encouragement and distraction. Thanks especially to Dr Katie Asplin for her support, but most of all for her constant injections of humour and entertainment, which lightened up long days in the laboratory and tedious trips to the abattoir.

I would like to thank my family and friends for their unfaltering support and encouragement, especially Rachael Stone, Brenda Rooker, Janet Walker, Pauline Richey and Alexandra Harvey. I am eternally grateful to my mother, Linda, and my late father, Roy, for their constant love and support, and for instilling their passion for animal welfare in me. Thanks especially to my mother whose strength, independence and humour in the face of adversity have been inspirational.

Lastly, my wholehearted gratitude and affection go to my husband Simon, whose enduring patience, love and understanding has gone above and beyond the call of duty.

This thesis is dedicated to the memory of my father,

Royden William Clutterbuck, MRCVS.

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Abbreviations

°C	Degrees Celsius
%	Percent
1-D	One-dimensional
3-D	Three-dimensional
15-ΔPGJ$_2$	15-deoxy-delta(12,14)prostaglandin J$_2$
µg	Microgram, weight
µl	Microlitre, volume
µM	Micromolar, concentration
µm	Micron, length
ACLT	Anterior cruciate ligament transection
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGE	Advanced glycation end products
ANOVA	Analysis of variance
AP-1	Activator protein-1
ASU	Avocado/soybean unsaponifiables
bFGF	basic fibroblast growth factor
CHAD	Chondroadherin
CILP-1	Cartilage intermediate layer protein -1
CLUS	Clusterin

cm	Centimetre, length
CO_2	Carbon dioxide
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
CP	Commercial product
cPGES	cytosolic prostaglandin E synthase
$cPLA_2$	cytoplasmic phospholipase A_2
Da	Dalton
DC	Detergent Compatible
DMEM	Dulbecco's modified Eagle's medium
DMMB	Dimethylmethylene blue
DMOAD	Disease modifying osteoarthritis drug
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin gallate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate

ESI	Electrospray ionisation
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
H_2O	Water
H_2O_2	Hydrogen peroxide
H & E	Haematoxylin and eosin
HCT	High capacity ion trap
HIF-1	Hypoxia-inducible factor 1
HIF-1α	Hypoxia-inducible factor 1 alpha subunit
H-PGDS	Hematopoietic prostaglandin D_2 synthase
HRP	Horseradish peroxidise
ICC	Ion charge control
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-1 binding protein
IκB	I kappa B kinase
IL	Interleukin
IL-1α	Interleukin-1alpha
IL-1β	Interleukin-1beta
IL-1R	Interleukin-1 receptor

IMS	Industrial methylated spirit
kDa	kilo Dalton
L	Litre, volume
LC	Liquid chromatography
LDS	Lithium dodecyl sulphate
L-PGDS	Lipocaline prostaglandin D_2 synthase
LPS	Lipopolysaccharide
M	Molar, concentration
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MCP	Metacarpophalangeal
mg	Milligram, weight
min	Minute, time
ml	Millilitre, volume
mm	Millimetre, length
mM	Millimolar, concentration
mPGES-1	microsomal prostaglandin E synthase-1
mPGES-2	microsomal prostaglandin E synthase-2
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MTP	Metatarsophalangeal

MW	Molecular weight
NF-κB	Nuclear factor-kappa B
ng	Nanogram
nl	Nanolitre
NO	Nitric oxide
n.s.	Not significant
NSAID	Nonsteroidal anti-inflammatory drug
O_2	Oxygen
OA	Osteoarthritis
OCD	Osteochondritis dessicans
$ONOO^-$	Peroxynitrite
OSM	Oncostatin M
PADI4	Protein arginine deiminase type-4
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pg	Picogram
PGA_2	Prostaglandin A_2
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase

$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFS	Prostaglandin $F_{2\alpha}$ synthase
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PGIS	Prostaglandin I_2 synthase
PGJ_2	Prostaglandin J_2
PI	Propidium iodide
PL	Placebo
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear
PT	Plant extract cocktail
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
S.D	Standard deviation
SDS	Sodium dodecyl sulphate
S.E.M	Standard error of the mean
SLRP	Small leucine-rich repeat proteoglycan
SNP	Sodium nitroprusside

$sPLA_2$	soluble phospholipase A_2
SPS	Smart parameter setting
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing Tween-20
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling
TxA_2	Thromboxane A_2
TxAS	Thromboxane A synthase
V	Volts
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1. INTRODUCTION

Osteoarthritis (OA) is a disease of the articulating joint, associated with degeneration of the articular cartilage and pathological changes in the surrounding tissues (Brandt *et al.*, 2006). It has considerable welfare and economic consequences for both human and companion animals through increased pain, lack of mobility, reduced athletic performance and the cost of medical therapy. This chapter will introduce the main anatomical structures involved in OA, the pathology of the disease referring to literature from a variety of species including the horse, current medicinal therapy and alternative nutraceutical therapies. Current *in vivo* and *in vitro* models of OA will then be discussed before the aims of the thesis are stated.

1.1. Articular Cartilage

In the healthy vertebrate, articular cartilage covers the ends of the long bones of synovial joints. It is a mechanically resilient connective tissue that enables smooth articulation and load bearing whilst protecting the underlying bone during movement (Kuettner, 1992). Articular cartilage is an avascular, aneural and alymphatic tissue mainly consisting of a tough extracellular matrix (ECM). Chondrocytes are the sole cell type present in the articular cartilage and are interspersed within the matrix (figure 1) forming between 1-10% of total tissue volume, depending on joint location and species (Stockwell, 1971; Carney and Muir, 1988; Hunziker *et al.*, 2002).

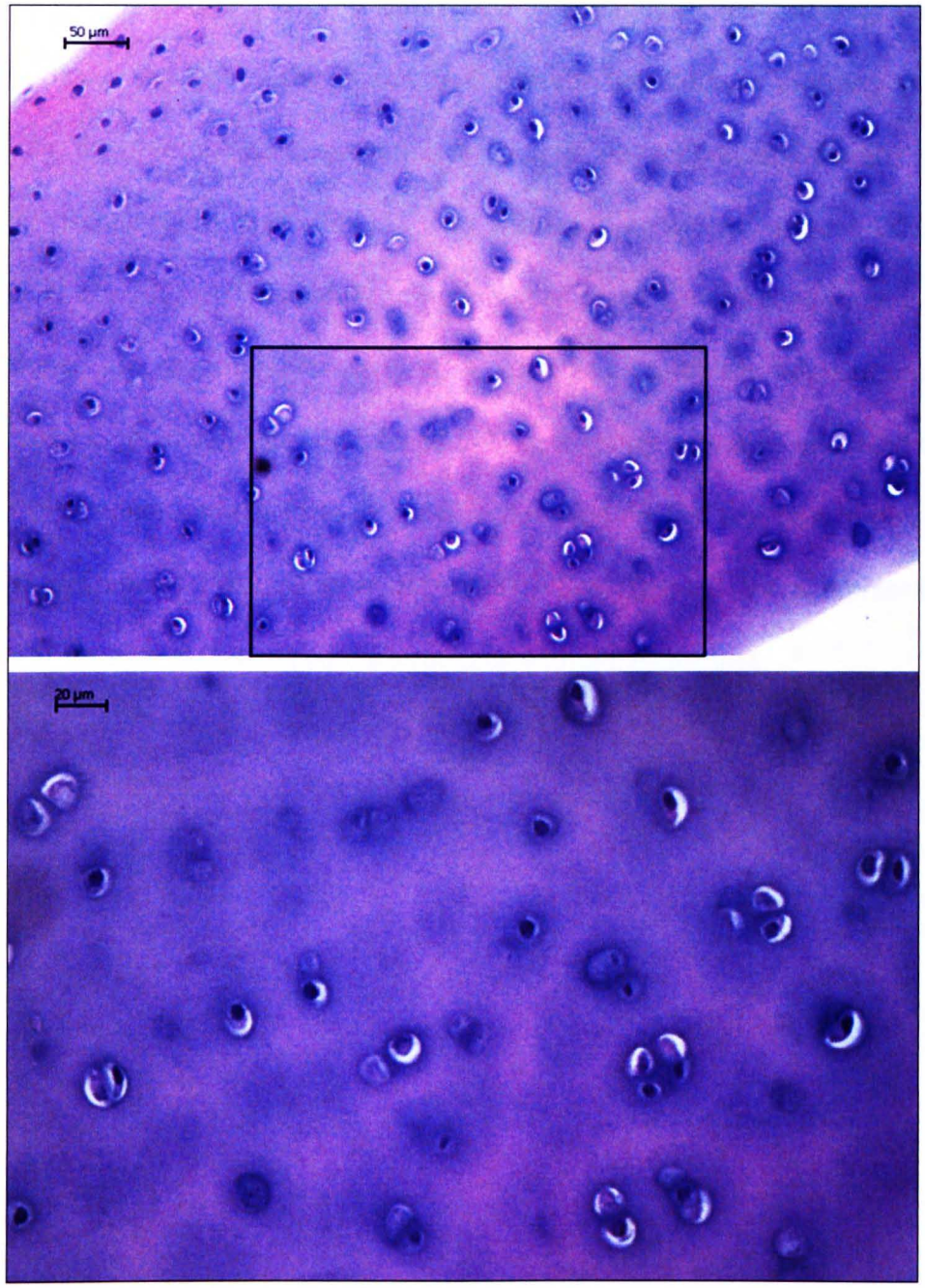

Figure 1. Haemotoxylin and eosin stained cross section of healthy equine articular cartilage

Cartilage taken from the left metacarpophalangeal joint of a young pony for this thesis. A. Photograph of a cross section of articular cartilage showing chondrocytes embedded within the lacunae of the matrix x200. B. represents a higher magnification (x400) of the box in A.

The ECM of articular cartilage is composed of collagens, predominantly collagen type II, which strengthen the matrix and restrain the osmotic swelling pressure caused by the hydrophilic aggregating proteoglycans (aggrecan), thereby giving cartilage viscoelasticity and allowing it to withstand compressive force (Kuettner, 1992; Li *et al.*, 2005). This ability to hydrate and resist compressive force is aided by sulphated glycosaminoglycans (GAGs), such as chondroitin sulphate and to a lesser extent keratan sulphate. Sulphated GAGs, together with non-sulphated GAGs such as hyaluronan, attach to their specific domains on the aggrecan molecule to form the aggregates (Watanabe *et al.*, 1998). See figure 2 for a representative diagram of the synovial joint and the structures within the cartilage.

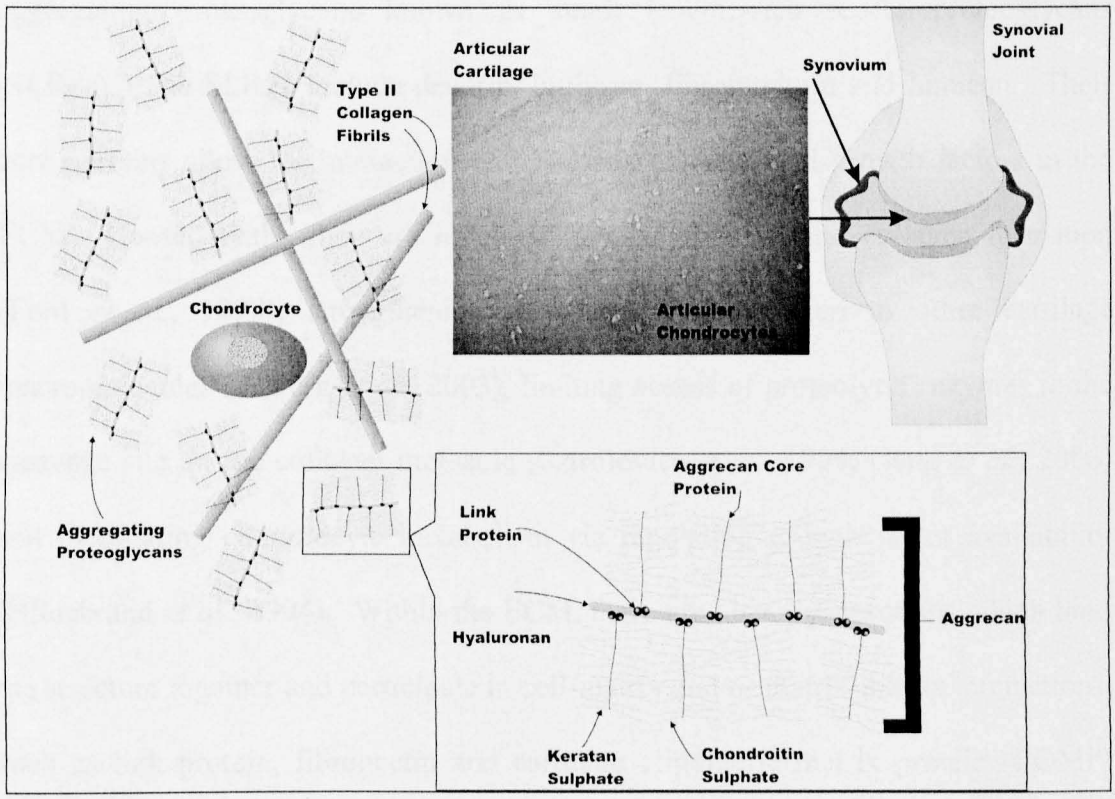

Figure 2. Structure of the synovial joint and the articular cartilage

Diagram highlights the main components of the articular cartilage extracellullar matrix; type II collagen and aggregating proteoglycans. Enlarged box identifies the individual components that make proteoglycans. Reproduced from Goggs et al. (2005) with permission of the authors.

In addition to the aggregating proteoglycans, the ECM also contains small non-aggregating proteoglycans known as small leucine-rich repeat proteoglycans (SLRPs). The SLRPs include decorin, biglycan, fibromodulin and lumican. Their core proteins allow for interaction with various collagen and growth factors in the ECM. Consequently, they are involved in regulating fibrillar collagen formation (Font *et al.*, 1998), strengthening the linkage of collagen to other cartilage macromolecules (Wiberg *et al.*, 2003), limiting access of proteolytic enzymes to the cleavage site on the collagen molecule (Sztrolovics *et al.*, 1999; Geng *et al.*, 2006) and modulating chondrocyte metabolism via regulating growth factor availability (Hildebrand *et al.*, 1994). Within the ECM, there are also glycoproteins which bind the structure together and participate in cell-matrix and/or matrix-matrix interactions, such as link protein, fibronectin and cartilage oligomeric matrix protein (COMP) (Hardingham, 1979; Rosenberg *et al.*, 1998; Roughley, 2001; Di Cesare *et al.*, 2002).

As cartilage ages, the composition and structure of the ECM components change. In humans and dogs ageing is thought to decrease the swelling capacity of cartilage through a reduction in proteoglycan size, as well as losing the ability to withstand and recover from compression through increased levels of keratan sulphate and hyaluronan and loss of chondroitin sulphate (Bayliss and Ali, 1978; Inerot *et al.*, 1978). With respect to equine cartilage, it is thought that the foal is born with biochemically uniform articular cartilage which adapts to weight bearing during the first months postpartum by decreasing water, DNA and GAG content and increasing collagen content (Brama *et al.*, 2000b). This collagen network remains stable as the horse ages, although non-enzymatic glycation increases (Brama *et al.*, 1999). Increased non-enzymatic glycation results in the production of advanced glycation end products (AGE) in the collagen which is known to increase collagen stiffness

(Verzijl *et al.*, 2002). In addition, the changes in the proteoglycan size seen in canine and human cartilage also occurs (Platt *et al.*, 1998). Thus, as cartilage ages, it becomes more brittle and less elastic.

Chondrocyte interactions with the ECM are mediated by the presence of transmembrane-receptors and proteoglycans, namely integrins and NG2 on their primary cilia which are involved in the signalling processes required for ECM synthesis and maintenance (McGlashan *et al.*, 2006). Consequently, normal cartilage matrix turnover is governed by the chondrocytes, the rate of which is determined by various stimuli. For example, growth factors such as insulin-like growth factor (IGF)-II can stimulate deoxyribonucleic acid (DNA) and GAG synthesis in adult equine cartilage *in vitro* via their mitogenic effects on the chondrocytes (Henson *et al.*, 1997; Davenport-Goodall *et al.*, 2004). In contrast, cytokines such as interleukin-1beta (IL-1β) can contribute to cartilage degeneration by decreasing GAG synthesis by the chondrocytes (Ikebe *et al.*, 1986). Growth factors and cytokines can regulate each other to maintain the homeostasis of ECM turnover. For example, IL-1β can increase IGF-I secretion and IGF-1 receptor production (Matsumoto *et al.*, 1994), thereby promoting cartilage anabolism. IGF-I can also up-regulate the production of IL-1 receptor (IL-1R)-II, which acts as a decoy receptor for IL-1alpha (IL-1α) and IL-1β (Wang *et al.*, 2003), to regulate IL-1-mediated catabolism. However, IL-1β can also increase IGF-1 binding protein (IGFBP)-3 which modulates IGF-1 activity (Olney *et al.*, 1995). Thus, the cytokines and growth factors regulate each other to govern cartilage turnover.

Various intracellular and extracellular proteinases are thought to facilitate cartilage depletion, notably the matrix metalloproteinases (MMPs) which degrade ECM proteins such as collagen, and the ADAMTSs (A Disintegrin and Metalloproteinase

with ThromboSpondin motifs) which primarily degrade aggrecan (Cawston *et al.*, 1999). The MMPs identified to date are grouped according to their substrate specificity, e.g. the collagenases MMP-1, MMP-8, MMP-13 and MMP-18, which degrade collagen. However, these substrate targets often overlap meaning that together they can degrade a variety of ECM proteins (Sternlicht and Werb, 2001). This substrate diversity is illustrated in figure 3

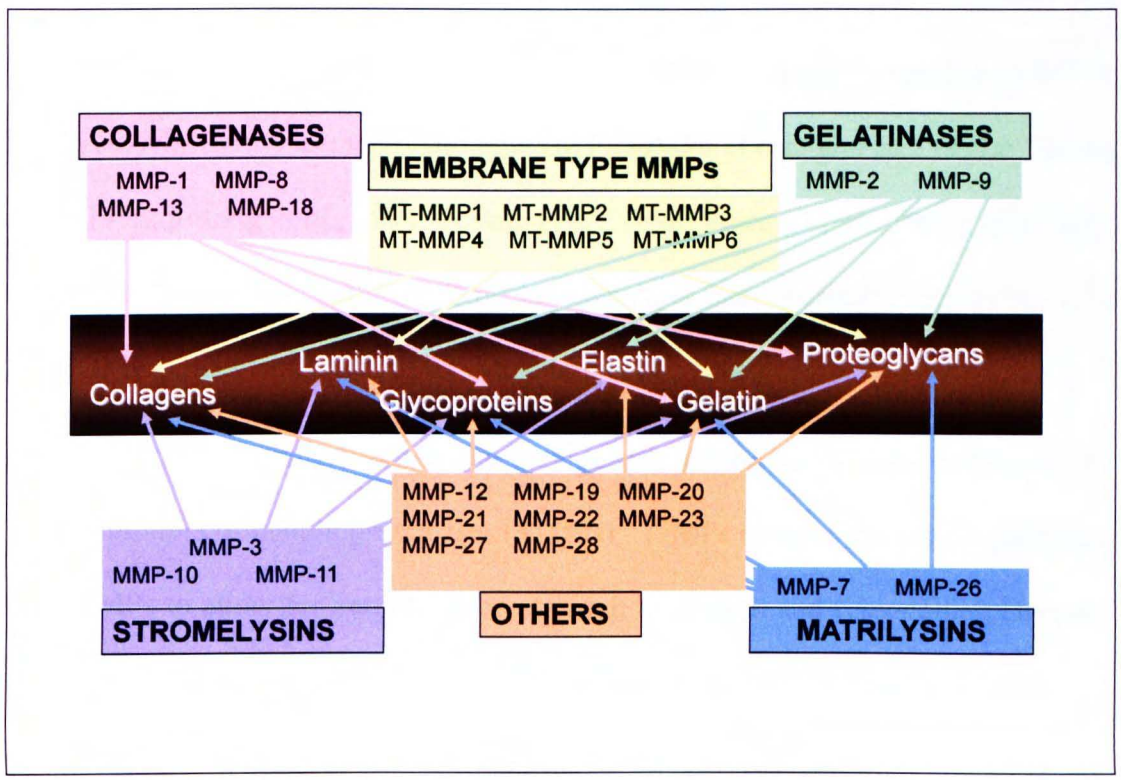

Figure 3. Schematic illustration of the substrate diversity of groups of known matrix metalloproteinases (MMPs)

The brown structure represents an extracellular matrix (ECM) containing a selection of labelled components which form the substrates for many MMP groups as highlighted by the arrows (Clutterbuck et al., 2010).

Similarly, ADAMTS enzymes are grouped according to their main substrates. For example, the hyalectanases: ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9, ADAMTS-15 and ADAMTS-20 that cleave the hyalectan (hyaluronan-binding proteoglycan) aggrecan, and the procollagen N-propeptidases: ADAMTS-2, ADAMTS-3 and ADAMTS-14, that cleave the amino peptides of type I, type II and type III procollagens (Jones and Riley, 2005). ADAMTS-7 and ADAMTS-12 specifically associate with and degrade COMP (Liu *et al.*, 2006a; Liu *et al.*, 2006b).

Although several ADAMTS enzymes can degrade aggrecan, it is also degraded by a variety of MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13 and membrane type (MT)-MMP1 (Fosang *et al.*, 1991a; Fosang *et al.*, 1992; Fosang *et al.*, 1994; Fosang *et al.*, 1996; Fosang *et al.*, 1998). Similarly, some ADAMTSs also degrade ECM substrates other than aggrecan, such as biglycan and COMP (Dickinson *et al.*, 2003; Melching *et al.*, 2006).

The chondrocytes also synthesise the endogenous inhibitors of these proteases, the tissue inhibitors of metalloproteinases (TIMPs). TIMPs exist in dynamic equilibrium with MMPs to allow for natural processes such as growth and remodelling (Joronen *et al.*, 2000), thereby maintaining the homeostasis of the articular cartilage ECM.

Cartilage homeostasis is altered with age and exercise. Transforming growth factor-β (TGF-β) messenger ribonucleic acid (mRNA) production by the articular chondrocytes of immature foals increases until around seven months to one year old, when expression declines concomitantly with a decreased responsiveness of the chondrocytes to TGF-β and reduced proteoglycan synthesis (Iqbal *et al.*, 2000; Nixon *et al.*, 2000). Thus, although the anabolic ability of the chondrocytes increases as the horse develops, once maturity is reached, this capacity is slowly reduced.

In young horses, regular low impact exercise, such as that achieved by foals kept out on pasture, is necessary for normal joint development (van de Lest *et al.*, 2002). Low level exercise postpartum stimulates the chondrocytes to synthesise a heterogeneous ECM, providing a stable collagen framework for later life, when collagen turnover decreases (Brama *et al.*, 2000b). Strenuous exercise, however, is associated with lesion development in the articular cartilage and loosening of the collagen network in young Thoroughbred horses (Brama *et al.*, 2000a). A 24-month training programme of young Standardbred trotters showed that increasing exercise intensity increased collagen degradation and reduced COMP release due to reduced synthesis of matrix proteins (Skioldebrand *et al.*, 2006). Thus, excessive loading on joints along with ageing can cause disruption to the homeostasis of the ECM, by reducing the anabolic capacity of the chondrocytes and increasing catabolic mediator production.

1.2. Osteoarthritis

OA is a degenerative disease involving degradation of the articular cartilage along with osteophyte formation, thickening of the subchondral bone plate and inflammation of synovium (Martel-Pelletier, 2004). It is thought that all the joint components can contribute to the development of the disease, namely the articular cartilage, the synovium, and subchondral bone (Samuels *et al.*, 2008). In addition, the joint ligaments, peri-articular muscle and surrounding nerves can play contributory roles (Brandt *et al.*, 2006). Figure 4 shows photographs of the structural differences between a healthy articular joint and those in various stages of disease progression.

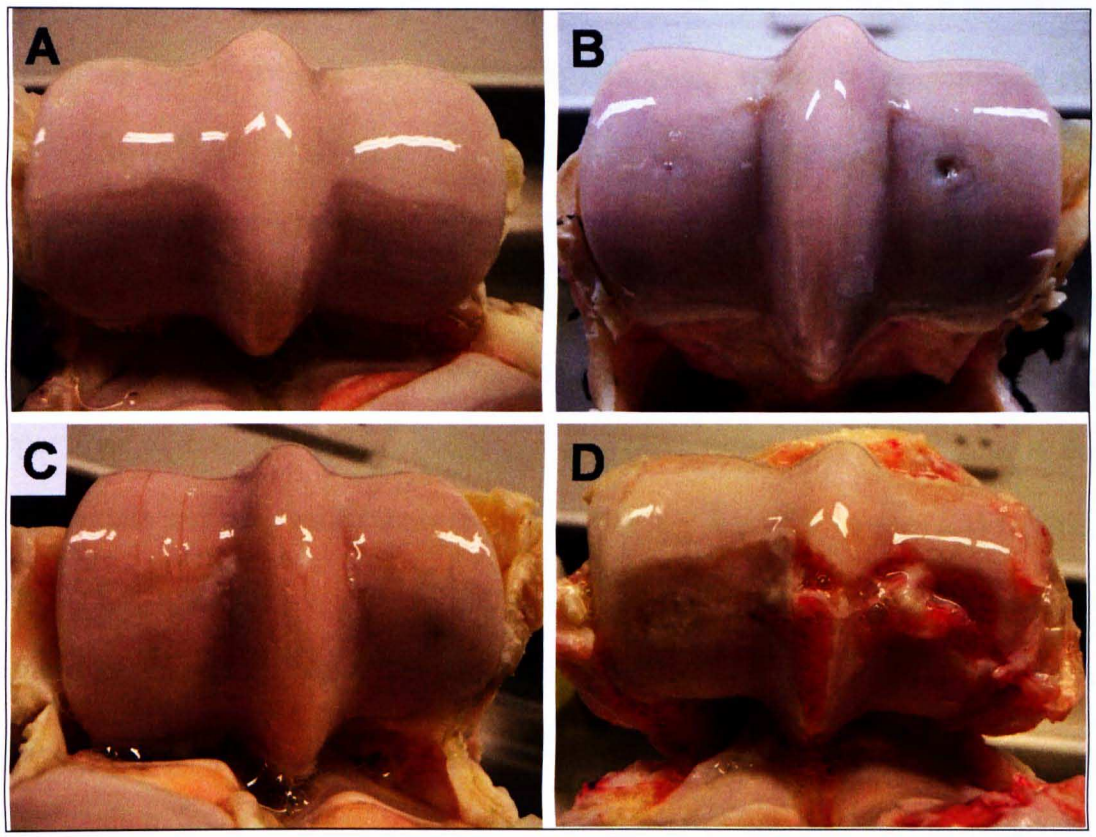

Figure 4. Equine metacarpophalangeal joints in health and disease

A is representative of a normal articular joint surface. The cartilage is macroscopically healthy, featuring smooth articular surfaces with even colouring and free of irregularities. The synovial fluid is pale and transparent and the synovium is not inflamed. ***B, C and D*** *are examples of unhealthy cartilage.* ***B*** *shows a patchy and irregular articular surface. The hole in the cartilage highlights a subchondral bone cyst.* ***C*** *depicts wear lines on the articular surface. The dark area reveals an underlying subchondral bone cyst and the synovium is thickened.* ***D*** *shows severe OA, with catastrophic cartilage degeneration exposing the subchondral bone. The synovium is thickened and inflamed with thick yellow synovial fluid.*

Unlike in normal cartilage where the chondrocytes are maintained in a pre-hypertrophic state, OA chondrocytes lose their phenotype, becoming hypertrophic, expressing type X collagen and eventually undergoing apoptosis (programmed cell death) (Sandell and Aigner, 2001). Although OA chondrocytes have increased expression of both anabolic and catabolic matrix genes (Aigner *et al.*, 2006), their catabolic ability outweighs their anabolic capacity, resulting in cartilage loss. This is attributed to zonal variation in matrix synthesis, with chondrocytes in the damaged upper zone of cartilage down-regulating matrix gene expression whilst those in the intact middle and deep zones show hyperactive matrix synthesis (Aigner and Dudhia, 1997). Consequently, increased synthesis in some zones cannot compensate for reduced synthesis in other zones, which, coupled with increased catabolism, results in cartilage loss. Thus, as OA progresses from mild to severe, there is a decrease in genes coding for transcription of collagen, failure to maintain the proteoglycan matrix and reduced ability of the chondrocytes to regulate apoptosis.

Osteoarthritic cartilage has been shown to have a higher proportion of apoptotic chondrocytes than normal cartilage (Blanco *et al.*, 1998). These apoptotic chondrocytes are associated with decreased proteoglycan content in human cartilage (Hashimoto *et al.*, 1998). Similarly, osteoarthritic equine articular cartilage has increasing numbers of apoptotic chondrocytes with higher histopathological degeneration scores as measured by both the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay (Kim *et al.*, 2003) and active caspase-3 immunohistochemistry (Thomas *et al.*, 2007). Caspases, notably caspase-3, are cysteine proteases involved in mediating nitric oxide (NO) induced chondrocyte apoptosis, being located in areas where progressive cartilage

degradation is occurring (Matsuo *et al.*, 2001). Caspases are also involved in the activation of the proteolytic MMPs.

Several proteolytic enzyme groups have been identified in OA cartilage, including collagenases, MMP-1, MMP-8 and MMP-13; gelatinases, MMP-2 and MMP-9; stromelysins, MMP-3, MMP-10 and MMP-11; membrane type MT1-MMP, and aggrecanases ADAMTS-4 and ADAMTS-5 (Goldring, 2000). The roles of ADAMTS-4 and ADAMTS-5 in OA are well established (Bayliss *et al.*, 2001; Moulharat *et al.*, 2004; Stanton *et al.*, 2005) but there has been some debate as to which is of greater importance (Huang and Wu, 2008). However, a recent study using normal and OA equine joints has suggested that both aggrecanases are important but in different joint structures, with ADAMTS-4 being produced by the chondrocytes in the cartilage and ADAMTS-5 by the synoviocytes in the synovium in the presence of elevated TNF-α (Kamm *et al.*, 2010).

Although the ADAMTSs and MMPs are the main focus for OA-treatment, another member of the matrixin family to which these enzymes belong, ADAM (a disintegrin and metalloproteinase)-8, has been identified as a potential target. ADAM-8 cleaves fibronectin, generating fibronectin fragments (Zack *et al.*, 2009). The proteolytic release of matrix protein fragments, such as fibronectin, can contribute to the pathogenesis of OA by up-regulating and activating MMPs (Homandberg and Hui, 1996; Forsyth *et al.*, 2002) and aggrecanases (Stanton *et al.*, 2002), as well as suppressing proteoglycan synthesis (Xie *et al.*, 1993). In addition to this, there is also less intact fibronectin, which is known to inhibit ADAMTS-4 activity (Hashimoto *et al.*, 2004). The exacerbation of matrix destruction by fibronectin fragments is attributed to the activation of various signalling pathways, which amplify the inflammatory response via stimulating chondrocyte chemokine and

cytokine release (Pulai *et al.*, 2005). Thus, inflammation plays a critical role in OA pathology.

1.3. Inflammation and Osteoarthritis

Although OA is defined as a non-inflammatory arthropathy, inflammation has a role in its pathogenesis (Hedbom and Hauselmann, 2002). Trauma to the components of the synovial joint, such as the synovium, articular cartilage and subchondral bone can initiate the production of many inflammatory and destructive mediators (figure 5).

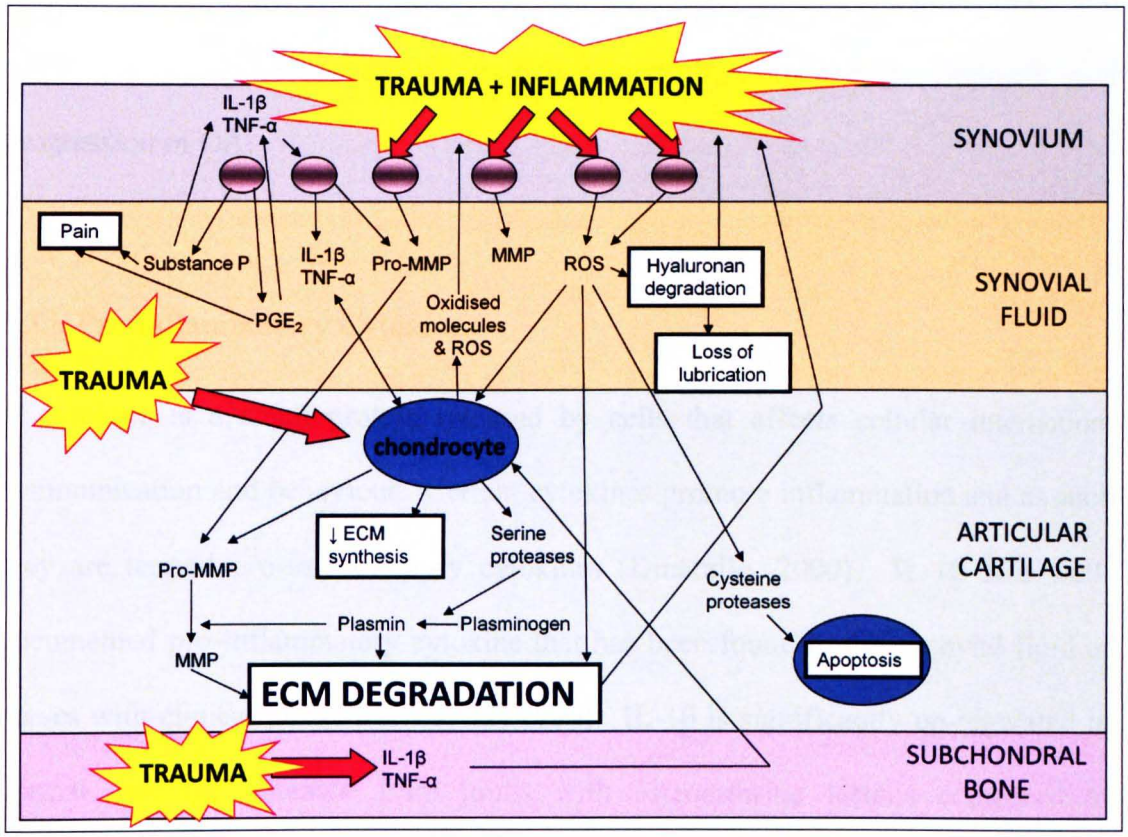

Figure 5. Molecules and structures involved in osteoarthritis of the articular joint

Pro-inflammatory cytokines interleukin-1beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) can arrive at the synovium from the circulation, or be produced by the synoviocytes, chondrocytes and subchondral bone tissue in response to trauma or inflammation. Production of reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and prostaglandin E_2 (PGE_2) promotes inflammation and cartilage extracellular matrix (ECM) degradation. At the same time ECM synthesis by the chondrocytes is reduced, chondrocytes die by apoptosis and serine proteases aid the conversion of plasminogen to plasmin, which activates latent MMP (adapted from Goodrich and Nixon (2006)).

Therefore, it can be seen that inflammation and cartilage destruction are intricately linked, and that pro-inflammatory cytokines, prostaglandins, neuropeptides and reactive oxygen species (ROS) oxidation contribute to the development and progression of OA.

1.3.i. Pro-inflammatory Cytokines

A cytokine is a small protein released by cells that affects cellular interaction, communication and behaviour. Certain cytokines promote inflammation and as such they are termed pro-inflammatory cytokines (Dinarello, 2000). IL-1β is a well-documented pro-inflammatory cytokine that has been found in the synovial fluid of horses with clinical OA (Morris *et al.*, 1990). IL-1β is significantly up-regulated in normal cartilage obtained from joints with osteoarthritic lesions compared to cartilage from healthy joints (Weaver *et al.*, 2006). This correlates with *in vivo* findings in the human field where IL-1β and TNF-α are significantly up-regulated in the synovial fluid of patients with early OA (Benito *et al.*, 2005). *In vitro*, the addition of pro-inflammatory cytokines such as IL-1β and TNF-α to equine chondrocytes and synovial fibroblasts stimulates the expression of MMPs and cyclooxygenase (COX)-2 which promote inflammation through prostaglandin production (Richardson and Dodge, 2000; Tung *et al.*, 2002). IL-1β and TNF-α are commonly cited in the literature as being involved in cartilage degradation, however, there are many other cytokines which are thought to contribute to the progression of OA. For example, cytokines such as IL-17 synergise with other cytokines such as IL-1, TNF-α, oncostatin M (OSM) and IL-6 to cause collagen degradation (Koshy *et al.*, 2002), as well as enhancing inflammation and destruction independently of TNF-α (Koenders *et al.*, 2006).

Transcription factors are intricately involved in up-regulating cytokines as well as mediating their effects. TNF-α inhibits link protein and collagen type II gene expression in chondrocytes via the nuclear factor-κB (NF-κB) and MEK1/2 pathways (Seguin and Bernier, 2003). IL-1β also stimulates the NF-κB pathway as well as the activator protein-1 (AP-1) pathway causing the down-regulation of collagen II and aggrecan expression and increased expression of MMPs (Martin *et al.*, 2005). In fact NF-κB has been described as the link between inflammation and joint hyperplasia in arthritis (Miagkov *et al.*, 1998). Thus, in an inflammatory state, cytokine activity can activate transcription factors, which can reduce cartilage synthesis as well as promoting cartilage degeneration by increasing MMP expression. In addition, chondrocytes cultured with IL-1β, show increased apoptosis in comparison to controls (Heraud *et al.*, 2000). Thus cytokines not only contribute to inflammation-associated cartilage degeneration, but they also reduce the reparative capacity of the chondrocytes by decreasing synthesis of appropriate matrix proteins and increasing cell death.

1.3.ii. Prostaglandins

Prostaglandins are eicosanoid lipid mediators that are involved in numerous homeostatic biological functions and inflammation (Funk, 2001). They are produced from the COX oxidation of arachidonic acid from cell membrane phospholipids (figure 6).

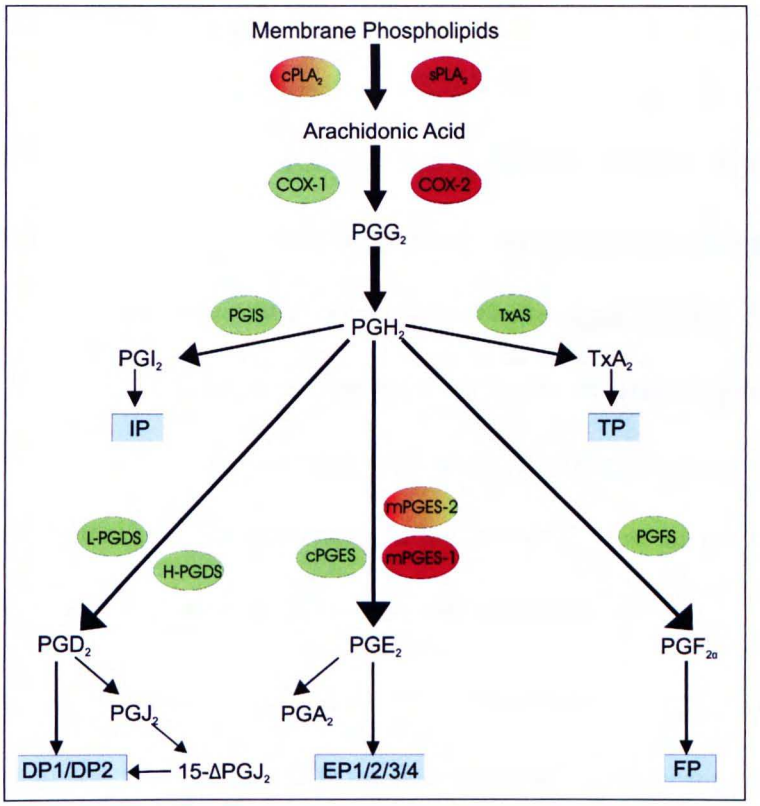

Figure 6. Prostaglandin synthesis pathway

The release of arachidonic acid from membrane phospholipids and its conversion to prostaglandins occurs by a sequence of enzymatic reactions involving soluble (s) or cytoplasmic (c) phospholipase A_2 (PLA_2) and cyclooxygenase (COX)-1 or -2. Constitutive enzymes are shown in green, inducible enzymes are in red, red and green together show enzymes that are both constitutive and inducible and specific receptors are in blue. The resulting prostaglandin H_2 (PGH_2) is converted by specific prostaglandin synthases into various prostaglandins. Prostaglandin I_2 (PGI_2) is converted by PGI synthase (PGIS) and acts via the IP receptor. Prostaglandin D_2 (PGD_2) is generated by hematopoietic and/or lipocaline PGD_2 synthases (H-PGDS, L-PGDS) where it either reacts with the DP1 or DP2 receptor, or is dehydrated to form prostaglandin J_2 (PGJ_2), which can be further converted to PGJ_2 derivatives such as 15-deoxy-delta(12,14)PGJ_2 (15-ΔPGJ_2). Prostaglandin E_2 (PGE_2) is derived from PGE synthases (PGESs), either cytosolic (c)PGES, microsomal (m)PGES-1, or mPGES-2. PGE_2 can either interact with various EP receptors (1-4) or become dehydrated to form prostaglandin A_2 (PGA_2). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is formed from PGF synthase (PGFS) and reacts with the FP receptor. Thromboxane A synthase (TxAS) converts PGH_2 into thromboxane A_2 (TxA_2) which interacts with the TP receptor (Stichtenoth et al., 2001; Goldring and Berenbaum, 2004; Kojima et al., 2004; Sampey et al., 2005; Scher and Pillinger, 2005; Komoto et al., 2006; Maicas et al., 2010).

The constitutively expressed COX-1 isoform is involved in the production of thromboxane A_2 (TxA_2), prostaglandin I_2 (PGI_2) and prostaglandin E_2 (PGE_2) in normal physiological processes, whereas the inducible COX-2 isoform releases proteases, prostaglandins and other inflammatory mediators in response to various stimuli, leading to further inflammation and pain (Steinmeyer, 2000). However, it is now known that both COX isoforms are involved in pathological and non-pathological processes such as arthritis and ulcer healing (Iniguez *et al.*, 1998; Brzozowski *et al.*, 2001), which has changed the classical view of the COX-1 being purely a housekeeping enzyme and COX-2 an inflammatory enzyme.

PGE_2 is the major eicosanoid involved in the pathogenesis of OA. It is found in increased levels in the synovial fluid from degenerative joints compared to normal joints in humans (Sahap Atik, 1990) and horses (Gibson *et al.*, 1996). *In vitro*, PGE_2 is produced in significantly higher amounts by OA chondrocytes compared to normal chondrocytes, both alone and in response to IL-1β stimulation (Jacques *et al.*, 1999). In addition, synovial COX-2-derived PGE_2 is associated with increased cartilage degradation in response to IL-1β-treated synovium and cartilage co-cultures (Hardy *et al.*, 2002). PGE_2 is thought to mediate this degradation via the EP4 receptor to increase MMP-13 and ADAMTS-5 levels (Attur *et al.*, 2008). It also contributes to the inflammatory pain associated with OA via sensitising peripheral nerve endings (Schaible and Schmidt, 1988). Therefore, PGE_2 contributes to both inflammatory processes and cartilage destruction in OA, as well as contributing to the pain associated with the disease.

Other prostaglandins, such as prostaglandin D_2 (PGD_2) are also found in OA cartilage, and are produced by chondrocytes in response to IL-1β (Zayed *et al.*, 2008b). However, their presence can be associated with an anti-inflammatory/ anti-

catabolic response. For example, PGD_2 reduces MMP-1 and MMP-13 production by IL-1β-stimulated chondrocytes via the DP1 receptor (Zayed *et al.*, 2008a). 15-deoxy-Delta prostaglandin J_2 (15-ΔPGJ_2) is derived from PGJ_2 which itself is a dehydration product of PGD_2 (Scher and Pillinger, 2005). 15-ΔPGJ_2 has been shown to block IL-1β-induced COX-2 expression in human synovial fibroblasts (Farrajota *et al.*, 2005), thereby suggesting it is a prostaglandin with anti-inflammatory and anti-degradative properties.

1.3.iii. Substance P and Derived Factors

Substance P is a neuropeptide involved in the perception of pain (nociception) and inflammation. It is released from peripheral nerve endings in response to various inflammatory mediators such as prostacyclin and pro-inflammatory cytokines (Hingtgen and Vasko, 1994; Malcangio *et al.*, 1996). Substance P can also be released from inflammatory cells such as eosinophils and macrophages (Aliakbari *et al.*, 1987; Bost *et al.*, 1992).

In addition, osteoarthritic synovial fibroblasts cultured *in vitro* have been shown to release substance P into the joint cavity in response to TGF-β and basic fibroblast growth factor (bFGF) (Inoue *et al.*, 2001). Thereby suggesting that substance P can be released from a variety of sources into tissues where it contributes to the inflammatory process.

Substance P causes local extravasation and vasodilation (Lembeck and Holzer, 1979), as well as having a role in the chemo-attraction of neutrophils to the site of release (Perretti *et al.*, 1993). In addition, substance P also potentiates the release of inflammatory mediators such as cytokines, oxygen (O_2) radicals and arachidonic acid

derivatives, thus amplifying the inflammatory response (Brain, 1997; O'Connor *et al.*, 2004).

Both substance P and PGE_2 have been found in higher concentrations in the synovial fluid of osteoarthritic joints in horses classified as being lame for over one month, compared to normal joints in horses with no history of lameness (Kirker-Head *et al.*, 2000). From clinical assessments of lame horses correlating inflammatory mediators in the synovial fluid with clinical joint pain, it has been suggested that substance P is associated with joint pain whereas PGE_2 reflects joint pathology, though it is implicated in the induction of pain (de Grauw *et al.*, 2006). Numerous other synovial derived neuropeptides such as corticotropin-releasing factor, urocortin and vasoactive intestinal peptide are also implicated in OA and have recently been reviewed (Sutton *et al.*, 2009).

1.3.iv. Reactive Oxygen Species

ROS include NO, superoxide anion and their derivative radicals, peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2), and they have been postulated as contributors to cartilage homeostasis due to their involvement in intracellular signalling pathways and in modulating gene expression (Henrotin *et al.*, 2003a). ROS have even been shown to exert anti-inflammatory activity by down-regulating expression of inflammatory genes by chondrocytes (Mathy-Hartert *et al.*, 2003). However, ROS are molecules that can oxidise and damage, all classes of biomolecules (Cheeseman and Slater, 1993), hence their implication in degenerative diseases. They are also able to activate signalling cascades and transcription factors which up-regulate MMP expression (Nelson *et al.*, 2006) and mediate apoptosis (Del

Carlo and Loeser, 2002), thus contributing to cartilage degeneration. In support of this, ROS activity has been found in the synovial fluid of horses with joint disease, i.e. those undergoing arthroscopic surgery for traumatic joint injury or those with osteochondritis dessicans (OCD) (cracking of the articular cartilage due to the failure of the subchondral bone to form properly) (Dimock *et al.*, 2000).

Superoxide anion has been shown to mediate IL-1β-induced NF-κB activation in bovine chondrocytes (Mendes *et al.*, 2003), which leads to the up-regulation of several catabolic genes as previously explained. The catabolic actions of NO include MMP activation (Murrell *et al.*, 1995) chondrocyte apoptosis due to caspase activation and reduced proteoglycan synthesis (Taskiran *et al.*, 1994). However, NO also has a protective effect. NO has been shown to prevent apoptosis by inhibiting caspase-3 activation (Kim *et al.*, 1997), as well as protecting cells from oxidative damage and enhancing proteoglycan synthesis under hypoxic conditions (Matsushita *et al.*, 2004). NO also increases the synthesis of hyaluronan which can remove superoxide anion ROS (Hashimoto *et al.*, 2006). NO can also be anti-inflammatory as it can reduce PGE_2 production by IL-1β-stimulated chondrocytes (Henrotin *et al.*, 1998). Therefore, it is thought that at low levels NO has an anti-catabolic effect but that this becomes catabolic as production persists.

1.4. Inflammation and Predisposing Factors to Osteoarthritis

There are many proposed risk factors for the development of OA including genetic factors, mechanical stress, trauma and damage to the articular cartilage and/ or subchondral bone (Muraoka *et al.*, 2007). Ageing is also a factor, as it is thought to reduce the ability of chondrocytes within cartilage to respond appropriately to

mechanical and oxidative stresses (Loeser, 2009). In horses, links have been drawn between excessive loading of joints during strenuous exercise regimens and cartilage degeneration (Murray *et al.*, 1999). Thus, the athletic nature of the horse and the performance sports for which it is used mean that it is in a high risk category for developing the condition. Consequently OA is observed in racehorses (Fubini *et al.*, 1999), team-roping horses (Dabareiner *et al.*, 2005), Warmbloods (Penell *et al.*, 2005) and Arabians (Malone *et al.*, 2003). However, certain risk factors associated with OA such as ageing (Cantley *et al.*, 1999) and conformation (Clegg *et al.*, 2001), indicate that the disease also impacts on non-performance horses.

Poor anatomical conformation, for example joint malalignment, can cause uneven loading over the joint surface, which predisposes the joint to inflammation. It has been shown that, IL-β, TNF-α, MMP-3 and fibronectin are up-regulated in canine articular cartilage two weeks after impact damage (Pickvance *et al.*, 1993). Although this effect is transient, increased pressure or repetitive loading on the joint in performance horses and in those with poor conformation, may sustain this inflammatory response. In addition, a single impact load increased GAG loss and caspase-9-induced cell death in equine cartilage explants *in vitro* (Huser and Davies, 2006; Huser *et al.*, 2006). Therefore, repeated, excessive or abnormal forces on articular cartilage can stimulate both inflammatory and degradative processes involved in the development of OA.

Thus, many inflammatory mediators are involved in the development and progression of OA, as well as the clinical manifestation of pain. Consequently, there are a variety of drugs are used to alleviate these symptoms.

1.5. Conventional Medical Therapy

In principle, the ideal therapeutic agent would target multiple processes of OA, i.e reducing inflammation and catabolism, whilst promoting cell survival and matrix anabolism to aid repair, in the three main joint structures: the cartilage, synovium and subchondral bone (Qvist *et al.*, 2008). Such an agent may be termed a chondroprotective, i.e. a substance that protects the articular cartilage during the process of OA, or a disease modifying OA drug (DMOAD), i.e. a substance that can change the course of a disease process (Verbruggen, 2006). However, no safe and effective DMOADs have been developed by the pharmaceutical industry to date (Le Graverand-Gastineau, 2010).

Current therapeutic drugs used in the treatment of OA in humans and companion animals only reduce the clinical signs, i.e. inflammation and pain (Trumble, 2005; Qvist *et al.*, 2008). These anti-inflammatory and analgesic drugs serve to alleviate patient discomfort, and may indirectly improve joint function via improving mobility and muscle tone to support the joint. Popular classes of drugs in conventional medical therapy include the nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids.

1.5.i. Nonsteroidal Anti-inflammatory Drugs

NSAIDs are widely used in the treatment of human and veterinary OA. They prevent prostaglandin synthesis by inhibiting the COX enzymes that catalyse their production (Meade *et al.*, 1993). They have also been shown to protect against chondrocyte cell death (Mukherjee *et al.*, 2001b).

The anti-inflammatory effects of NSAIDs have been attributed to COX-2 inhibition. NSAIDs targeting COX-1 and non-specific NSAIDs targeting both isforms are thought to cause gastrointestinal ulceration (Wolfe *et al.*, 1999; Bombardier *et al.*, 2000). Thus, COX-2 inhibitors were developed to reduce the risk of adverse gastric side effects (Lazzaroni and Bianchi Porro, 2004). However, in humans, adverse cardiovascular and nephrotoxic side effects have been associated with the use of COX-2 inhibitors (Mukherjee *et al.*, 2001a; Fletcher *et al.*, 2006). Also, COX-2 has been shown to be important in resolving inflammation by synthesising anti-inflammatory prostaglandins (Gilroy *et al.*, 1999). Therefore, complete COX-1 and/ or COX-2 inhibition can be damaging.

In the equine veterinary field, there are a variety of NSAIDs used to treat OA including phenylbutazone, flunixin, ketaprofen, naproxen and carprofen, all of which display differing degrees of analgesia and reduction of inflammation (Goodrich and Nixon, 2006). However, despite their beneficial effects, they are non-selective NSAIDs, therefore it is unsurprising many of these NSAIDs have been associated with gastric ulceration and colitis in horses (Andrews and McConnico, 2009). Although this topic is controversial (Fennell and Franklin, 2009), gastrointestinal toxicity of NSAIDs has been reported in many animal species (Radi and Khan, 2006).

NSAIDs have been reported to have an inhibitory effect on normal cartilage turnover *in vitro*. For example, phenylbutazone treatment of healthy equine articular cartilage explants reduced proteoglycan loss (Jolly *et al.*, 1995), and proteoglycan synthesis (Beluche *et al.*, 2001). This may be detrimental to normal joint health as well as prolonging healing in damaged cartilage. It has been demonstrated *in vitro* that neither phenylbutazone nor flunixin show any inhibitory activity against MMP-2 and

MMP-9 (Clegg *et al.*, 1998) and that some NSAIDs may promote latent MMP-9 and MMP-1 production by inhibiting regulatory PGE_2 (Ito *et al.*, 1995; Takahashi *et al.*, 1997). Therefore, although NSAIDs may reduce pain and inflammation in the joint, they can have potentially undesirable side effects.

There are now more specific NSAIDs on the market, known as coxibs, which selectively inhibit COX-2 rather than COX-1, thereby reducing the risk of these side effects. In a recent study in horses, firocoxib, was found to have comparable clinical efficacy with phenylbutazone in horses with naturally occurring OA, and neither treatment showed any signs of toxicity over the relatively short study duration (Doucet *et al.*, 2008). It may be that no adverse side effects are observed when either selective or non-selective NSAIDs are administered at the correct dosage for relatively short periods. However, a recent survey on 1,403 horse owners and trainers in the USA conducted by the animal healthcare company, Merial Ltd, found that 82% used NSAIDs without consulting their veterinarian, and 8% used the incorrect dosage (Anon, 2009). Consequently, there is still potential for adverse side effects with these drugs. This warrants research into chondroprotective agents for either prevention of OA to avert the need for NSAID therapy or as adjuncts to NSAID therapy in animals with established OA to help repair the cartilage as the inflammation decreases.

1.5.ii. Glucocorticoids

Glucocorticoids, also known as corticosteroids, are commonly used in the treatment of inflammatory diseases due to their anti-inflammatory capabilities. Glucocorticoids bind to cytoplasmic glucocorticoid receptors which can down-

regulate inflammatory genes and up-regulate anti-inflammatory genes (Adcock *et al.*, 2004). Their main effects are thought to be mediated through interactions between the glucocorticoid receptors and the transcription factors NF-κB and AP-1 preventing them from binding to inflammatory genes and promoting the inflammatory process (Barnes and Karin, 1997). In addition, glucocorticoids can also increase IκBα inhibitory protein which traps NF-κB in an inactive complex (Auphan *et al.*, 1995). Thus, glucocorticoids exert their anti-inflammatory effects via decreasing cytokine expression.

Current glucocorticoids used in the treatment of equine OA include methylprednisolone acetate, betamethasone acetate, triamicinolane acetonide, isoflupredone and flumethasone (Goodrich and Nixon, 2006). Most glucocorticoids are administered intra-articularly in order to deliver the drug directly to the site of inflammation thereby reducing the dosage required. Intra-articular glucocorticoid injections have also been shown to suppress TNF-α release from blood monocytes for at least four days post injection (Steer *et al.*, 1998). Thus, it can be seen that glucocorticoids are effective anti-inflammatory drugs and, as such, seem an appropriate choice for OA therapy.

However, as with NSAIDs, there have been concerns over the other effects that glucocorticoids may exert. Glucocorticoids may contribute to cartilage depletion as indicated by increased aggrecan turnover and reduced collagen synthesis in the synovial fluid after three intra-articular methylprednisolone acetate injections given to healthy horses over 13 weeks (Robion *et al.*, 2001). Similarly, intra-articular triamicinolane acetonide increased aggrecan turnover and collagen type I and II cleavage whilst increasing collagen synthesis in injected and untreated contra-lateral joints, indicating systemic negative effects of glucocorticoid administration on

cartilage metabolism (Celeste *et al.*, 2005). This cartilage cleavage was attributed to the glucocorticoid inducing proteoglycan release into the synovial fluid rather than via MMP activity. Reports on the effects of glucocorticoids on MMP activity are mixed. A study using the synovium of human patients with OA found that glucocorticoids did not alter the expression of MMP-1, MMP-3, TIMP-1 or TIMP-2 (Young *et al.*, 2001). Whereas, glucocorticoid treatment of equine chondrocytes was found to reduce expression of MMP-1, MMP-3, MMP-13 and TIMP-1 (Richardson and Dodge, 2003). However, several differences may account for this contradiction. Firstly, the reaction of the synovium to cytokines may be different to that of the chondrocyte. Secondly, the equine study used healthy chondrocytes stimulated with cytokines to replicate the diseased state unlike the human study, which used actual diseased tissue. The study also used recombinant human cytokines rather than equine specific ones. These factors may account for the differences between results. However, work seems to suggest that glucocorticoids do exert a suppressive effect on MMPs and TIMPs by reducing their expression and activity in cytokine treated chondrocytes *in vitro* (Sadowski and Steinmeyer, 2002). Despite reducing MMP activity, there is also a decrease in proteoglycan concentrations of explants treated with dexamethasone (Stove *et al.*, 2002). Thus, the effects of glucocorticoids on joint health may not all be beneficial. In addition, glucocorticoid use has been tenuously linked to the development of laminitis in horses via several potential pathways including glucose metabolism, insulin resistance, vasospasm and apoptosis (French *et al.*, 2000; Bailey and Elliott, 2007).

Although glucocorticoids are effective anti-inflammatories, neither they nor NSAIDs promote cartilage matrix synthesis. Consequently, natural food derived products, termed nutraceuticals, are being investigated for their chondroprotective ability as

potentially safer joint health supportive products, to replace or use alongside existing anti-inflammatory and analgesic pharmacotherapy.

1.6. Nutraceuticals

The term 'nutraceutical' is a portmanteau of the words 'nutrition' and 'pharmaceutical', that was coined by Stephen De Felice to give an identity to the vague nomenclatural area of foods with medical benefits (De Felice, 1995; Brower, 1998). A nutraceutical is defined as a food (or part of a food) that provides medical or health benefits, including the prevention and/ or treatment of a disease. Thus the aiding in the prevention and/ or treatment of disease is what distinguishes nutraceuticals from functional foods and dietary supplements, which simply provide the body with substances needed for healthy survival (Kalra, 2003). There are several issues surrounding the nutraceutical and dietary supplement industry from concerns about bioavailability, supraphysiological dosing levels, negative interactions with prescription drugs, increased risk of toxicity and harmful interactions with other foods, to issues surrounding undeclared substances on packaging and the variability in content of the active ingredient in the manufactured product (Ridinger, 2007; Sadovsky *et al.*, 2008). In light of this, there is a recognised need for greater regulation by legislative bodies on a global scale to ensure product quality, accurate labelling and health claims (Goggs *et al.*, 2005; Gulati and Berry Ottaway, 2006; Kaushik and Kaushik, 2010; Pandey *et al.*, 2010). Despite these issues, nutraceuticals have great potential and consequently are a popular area of research, especially in terms of chondroprotection. Two of the most common nutraceuticals marketed as chondroprotective agents on the human and veterinary market are glucosamine and chondroitin sulphate.

1.6.i. Glucosamine

Glucosamine is a constitutive amino sugar of the GAGs found in the joint. Thus, its exogenous administration is thought to have a beneficial effect on cartilage regeneration in the joint (Bassleer *et al.*, 1998a). However, treatment of mouse chondrocyte cultures with glucosamine has shown that glucosamine does not stimulate chondroitin sulphate synthesis (Mroz and Silbert, 2003). Work with human chondrocyte cultures has also shown that glucose competitively inhibits exogenous glucosamine (Mroz and Silbert, 2004). Subsequent work with bovine chondrocytes has supported these findings by showing that glucosamine does not increase GAG synthesis (Qu *et al.*, 2006). Glucosamine is thought to inhibit NF-κB activation and the expression of COX-2, thereby preventing the synthesis of pro-inflammatory mediators and cartilage destruction (Largo *et al.*, 2003). Thus, it appears that glucosamine may exert its effects through preventing catabolic mediators rather than promoting cartilage anabolism. This is supported by the fact that both anabolic and catabolic genes are down-regulated in response to glucosamine treatment in human osteoarthritic cartilage explants (Uitterlinden *et al.*, 2006).

With regard to equine *in vitro* work, glucosamine is also not thought to have an anabolic effect, as it does not significantly increase GAG synthesis in equine cartilage explants, with or without IL-1α (Dechant *et al.*, 2005). However, in terms of catabolism, glucosamine hydrochloride (HCl) at 25mg/ml has been shown to reduce NO production, proteoglycan release and MMP activity in equine cartilage explants stimulated with either lipopolysaccharide (LPS) or recombinant human IL-1β (Fenton *et al.*, 2000). A subsequent study found that glucosamine HCl (1.5-50mM) had no effects on MMPs activated by culturing cartilage explants in LPS, but concentrations of 25mM and 50mM reduced MMP-1, -3, and -13 mRNA expression

in LPS-stimulated chondrocytes rather than inhibiting their activity (Byron *et al.*, 2003). Therefore, the reduced MMP activity may be attributed to less MMPs being available for activation rather than the glucosamine directly inhibiting activated MMPs. However, these studies found beneficial effects at relatively high glucosamine concentrations. Pharmokinetic studies with horses have shown that serum and plasma glucosamine HCl concentrations following oral administration are roughly 1μg/ml (~6μM) with maximum synovial fluid concentrations reaching only about 10% of serum/ plasma levels (Laverty *et al.*, 2005; Meulyzer *et al.*, 2008). Thus, physiologically achievable concentrations in the joint are roughly 0.1μg/ml or 0.6μM. Although one study reported a maximum plasma concentration of 10.6μg/ml following oral glucosamine HCl (Du *et al.*, 2004), this in theory would mean 1.06μg/ml glucosamine in the synovial fluid. An equine model of LPS-induced joint inflammation has suggested that glucosamine accumulates in inflamed joints, as opposed to non-inflamed joints, achieving maximal synovial fluid concentrations of roughly 0.42μg/ml (2.36μM) and 0.09μg/ml (0.19μM) respectively following nasogastric administration of 20mg/kg glucosamine HCl to horses (Meulyzer *et al.*, 2009). However, despite this, these values are still considerably lower than those tested in chondrocytes and explants *in vitro* as previously mentioned. A more recent study found that physiologically relevant concentrations between 0.1 and 20μg/ml had no effect on MMP-13 production by IL-1-stimulated chondrocyte and synoviocyte cultures, but significantly reduced mPGES production by chondrocytes and both mPGES and PGE_2 production by synoviocytes (Byron *et al.*, 2008). Thus, the effects of glucosamine may be primarily anti-inflammatory and act on other cells found in the joint not just the chondrocytes. It should be mentioned here that the experimental model of equine joint inflammation described earlier found that despite

increased glucosamine accumulation in the inflamed joints, no effect was seen on standard synovial fluid parameters of inflammation- namely white blood cell counts and total protein analysis (Meulyzer *et al.*, 2009). However, this does not necessarily mean there was no anti-inflammatory effect as no other indicators of inflammation were studied, such as PGE_2 or pro-inflammatory cytokine levels.

Glucosamine has received much attention in human medicine, but clinical trial results have often been conflicting and compromised by poor, biased study designs (Barclay *et al.*, 1998; Vlad *et al.*, 2007). In horses, oral glucosamine supplementation for walked or lunged horses did not significantly alter serum markers of joint and bone metabolism, namely keratan sulphate and osteocalcin, a non-collagenous protein secreted by osteoblasts (Fenton *et al.*, 1999). Similarly, oral glucosamine HCl given to Standardbred horses in race training did not significantly alter serum concentrations of keratan sulphate, osteocalcin or pyridinoline crosslinks of type I collagen (Caron *et al.*, 2002). These results suggest that, in the horse, glucosamine does not influence cartilage and bone metabolism.

The lack of effects *in vivo* could be due to the fact that it is difficult to achieve the concentrations that prove effective *in vitro*. The bioavailability of glucosamine HCl administered at clinically relevant doses to horses via nasogastric intubation is poor (~5.9%) and, although it can enter synovial fluid, transport into the joint cavity from the circulation is inefficient, resulting in concentrations 500 times lower than those reported to have chondroprotective effects *in vitro* (Laverty *et al.*, 2005). In addition, there is considerable variation in glucosamine content in commercial equine joint supplements. One study found that nine out of the 23 supplements tested failed to meet the label claims (Oke *et al.*, 2006). Therefore, poor bioavailability as well as poor quality supplements and varying recommended dosages means that it is

unsurprising that *in vivo* results haven't reflected the initial success seen in chondrocyte cultures. However, the aforementioned glucosamine trials used exercising healthy horses and assessed markers of bone and cartilage metabolism. If, as *in vitro* results suggest, glucosamine has a greater influence on inflammation rather than catabolism or anabolism of cartilage and bone, then studying the expression and production of inflammatory mediators around the joints in healthy and early OA horses may be more beneficial.

Past trials in humans and animals have generally concluded that glucosamine is well tolerated and is safe for long term use (Anderson *et al.*, 2005; Hathcock and Shao, 2007). Despite this, there have been concerns of glucosamine toxicity towards chondrocytes *in vitro*. One study reported impaired chondrocyte metabolism and a loss in cell viability in response to glucosamine at 6.5mg/ml (de Mattei *et al.*, 2002), whereas another observed cell death at 10mg/ml, although 5mg/ml was the next lowest concentration tested (Mello *et al.*, 2004). However, as previously mentioned, these concentrations are not likely to be achieved in the joint *in vivo* following oral glucosamine administration, therefore these risks are probably minimal.

1.6.ii. Chondroitin Sulphate

Chondroitin sulphate is an orally administered GAG naturally present in cartilage. It is a much larger molecule than glucosamine and its large molecular weight has raised queries regarding its absorption via the gastrointestinal tract. However, it is available in both a normal, 16.9 kilo Dalton (kDa), form and a low molecular weight, 8.0kDa, form which when fed to horses at 3g per day, were shown to have a bioavailability of 32.2% and 22% respectively, with both these forms being absorbed to a greater

extent than glucosamine administered at 125mg/kg (Du *et al.*, 2004). Thus, the bioavailability of orally administered chondroitin sulphate appears to be adequate, though whether the biological activity of chondroitin sulphate is retained through the digestive process remains to be determined.

Unlike glucosamine, chondroitin sulphate has shown less consistent results *in vitro*. In equine articular cartilage explants, glucosamine (1mg/ml) reduced LPS-induced NO production relative to controls, and at 0.5mg/ml reduced PGE_2 levels compared to controls, whereas chondroitin sulphate at 0.25mg/ml and 0.5mg/ml failed to produce any effects (Orth *et al.*, 2002). Similarly, IL-1β-induced mRNA levels of MMP-13 and aggrecanase-1 in equine chondrocyte pellet cultures were significantly reduced by glucosamine (10μg/ml) but no effect was seen in samples treated with chondroitin sulphate at concentrations of 5-50μg/ml (Neil *et al.*, 2005). In contrast to this, another study which extracted RNA from bovine cartilage explants after culturing, showed that chondroitin sulphate (20μg/ml) decreased IL-1β-mediated MMP-13, and aggrecanase 1 and 2 gene expression (Chan *et al.*, 2005a). However, these studies examined catabolic mediators of cartilage degradation, whereas the chondroprotective properties of chondroitin sulphate may be more attributed to promoting cartilage anabolism. For example, chondroitin sulphate has been shown to counteract the effects of IL-1β on human articular chondrocytes during the first 16 days of culture by reversing IL-1β-stimulated suppression of proteoglycan and type II collagen synthesis (at concentrations of 500-1000μg/ml and 100-1000μg/ml respectively) whilst decreasing PGE_2 production (500-1000μg/ml) (Bassleer *et al.*, 1998b). Thus, it may be that chondroitin sulphate is capable of exerting both an anti-catabolic and pro-anabolic effect, in addition to anti-inflammatory effects, or that it exerts species-specific effects. Whichever the case, live animal studies have

indicated that a combination of chondroitin sulphate and glucosamine may be more effective (Hanson *et al.*, 1997; Canapp *et al.*, 1999).

1.6.iii. Glucosamine and Chondroitin Sulphate Combined

Studies examining the effects of chondroitin sulphate and glucosamine on cartilage explants have found that using a combination of the two substances has a more beneficial effect on cartilage than either agent alone in live animal models and *in vitro* explants (Lippiello *et al.*, 2000; Orth *et al.*, 2002; Homandberg *et al.*, 2006)

Studies with bovine cartilage explants found that chondroitin sulphate and glucosamine (10μg/ml and 20μg/ml respectively) together, effectively suppressed the IL-1β-induced expression of inflammatory and proteolytic genes including NF-κB, COX-2 and MMP-13, whilst up-regulating TIMP-3 expression, implying that the combination of agents has anti-inflammatory and anti-catabolic properties (Chan *et al.*, 2006). Thus, *in vitro* results using a combination of chondroitin sulphate and glucosamine have been promising.

There are few *in vivo* equine studies examining the effects of combined chondroitin sulphate and glucosamine, and they have produced conflicting results. Daily oral supplementation for 26 days with glucosamine (10.8g) and chondroitin sulphate (3.6g) did not improve lameness score, stride length, carpal circumference, carpal flexion or synovial fluid protein levels in horses with chemically induced synovitis and degenerative joint disease compared to untreated controls (White *et al.*, 1994). Whereas horses given 9-12g of a combined glucosamine HCl/ chondroitin sulphate compound (Cosequin) twice a day for 6 weeks showed significant improvements in stride length, lameness grade and flexion test 2 weeks after the compound began to

be administered, though there was no further improvement after 4 weeks (Hanson *et al.*, 1997). An eight year study of 10 working show jumpers/ hunters found that oral supplementation with glucosamine and chondroitin sulphate (10g/day) reduced the mean number of distal tarsal joint injections per year from 1.7 to 0.85, and increased the time interval between injections from 6.8 months to 9.98 months to maintain soundness (Rodgers, 2006). The differences between criteria used to assess improvement in horses, limitations with regard to blinding, the use of appropriate controls, small study numbers and limited if any statistical analysis make comparative analysis difficult between these studies. However, despite this, the basic data from the *in vivo* trials combined with *in vitro* work provide encouraging evidence of the chondroprotective ability of glucosamine and CS.

There is still much scepticism on the ability of these agents either alone or in combination to act as chondroprotective agents *in vivo*, with bioavailability being one of the main criticisms (Ramey, 2005). However, the *in vitro* results with these agents on chondrocytes and in explant models, along with some basic *in vivo* trial data have been too effective to dismiss them and their potential role in the treatment of osteoarthritic conditions. Therefore, carefully designed trials on the efficacy and safety of these substances are needed to clarify their role as chondroprotective agents in the horse.

1.7. Plant Derived Nutraceuticals

Plant derived substances with anti-inflammatory properties are being investigated for their potential in the treatment of a range of human and veterinary diseases including OA. Ethnoveterinary medicine uses indigenous knowledge on animal health and can

be used to identify plants with potential therapeutic properties. This area has received so much interest that national data banks have been set up to record and preserve this information (Viegi *et al.*, 2003).

A review of ethnoveterinary medicines used for horses in Trinidad and British Columbia found a variety of plants were added in powdered form to horse feed for the treatment of arthritis and sore joints (Lans *et al.*, 2006), see table 1.

Scientific Name	**Common Name**	**Plant Part**	**Use**
Arctium lappa	Burdock	root	Arthritis
Capsicum sp	Cayenne	fruits	Joint pain
Curcuma longa	Turmeric	rhizome	Arthritis
Equisetum arvense	Horsetail	aerial parts	Arthritis
Filipendula ulmaria	Meadowsweet	whole plant	Arthritis
Glycyrrhiza glabra	Liquorice	root	Arthritis
Harpagophytum procumbens	Devils Claw	root	Arthritis
Lavendula sp	Lavender	flowers	Arthritis
Salix alba	White Willow	bark	Arthritis
Scutellaria baicalensis	Baical Skullcap	aerial parts, leaves	Arthritis
Scutellaria lateriflora	Skullcap	aerial parts, leaves	Pain
Silybum marianum	Milk Thistle	seed	Arthritis

Table 1. Plant remedies used in British Columbia for the treatment of arthritis and joint pain in horses adapted from Lans *et al.* (2006)

Many plant extracts have been investigated for their beneficial anti-inflammatory properties and anti-degradative properties in a variety of cell types, including chondrocytes. For example, the green tea polyphenol, epigallocatechin gallate

(EGCG), from the leaves of *Camellia sinensis* inhibits IL-1β-induced mRNA expression and enzyme activity of MMP-1 and MMP-13 in human chondrocytes (Ahmed *et al.*, 2004). Pre-treatment of chondrocytes with pomegranate extract inhibits the IL-1β-induced increase in MMP-1, MMP-3 and MMP-13 production (Ahmed *et al.*, 2005). Resveratrol from red grapes reduces IL-1β-stimulated MMP-3, MMP-9 and COX-2 expression, as well as chondrocyte apoptosis (Shakibaei *et al.*, 2008). The flavanoid, nobiletin, from *Citrus depressa* suppresses COX-2 mRNA expression but not COX-1, similar to the coxib drugs, as well as increasing TIMP-1 production in synovial fibroblasts (Lin *et al.*, 2003). Thus, some extracts can exert multiple anti-inflammatory and anti-degradative effects on cartilage *in vitro*.

The anti-catabolic activities of plant extracts on cartilage can be accompanied by pro-anabolic effects. For example, avocado/soybean unsaponifiables (ASU) can restore aggrecan synthesis in IL-1β-stimulated human OA chondrocytes, whilst concurrently inhibiting MMP-3 production (Henrotin *et al.*, 2003b). Some extracts may be more beneficial in combination as shown by a recent study combining ASU and EGCG which reduced COX-2 expression and PGE_2 production in IL-1β-stimulated equine chondrocytes (Heinecke *et al.*, 2010). In addition, the combination of resveratrol and the polyphenol curcumin, derived from turmeric, has been shown to reduce MMP-3, MMP-9 and COX-2 production by IL-1β-stimulated chondrocytes (Csaki *et al.*, 2009). Many of these plant extracts exert their anti-inflammatory actions through blocking the translocation and activation of the transcription factors, primarily NF-κB (Singh *et al.*, 2002; Ahmed *et al.*, 2004; Ahmed *et al.*, 2005; Gabay *et al.*, 2008; Csaki *et al.*, 2009). Figure 7 summarises the modulatory actions of some known plant extracts on the NF-κB pathway.

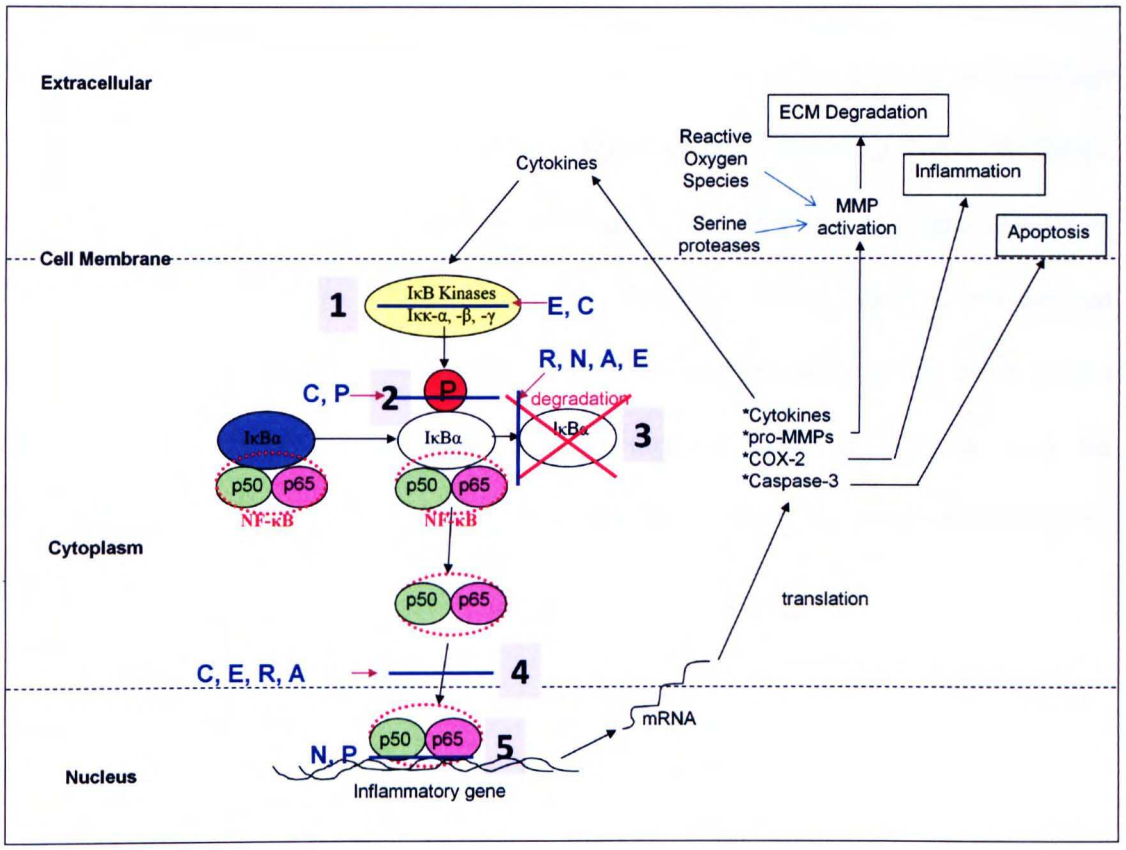

Figure 7. Areas for nutritional modulation of the nuclear factor-kappa B (NF-κB) pathway in arthritis

Activation of the NF-κB pathway is initiated by cytokines, such as IL-1β, binding to their receptors on the cell membrane. This starts an intracellular signal transduction cascade, numbered as follows; ***1****. Activation of cytoplasmic IκBα kinases (IKK)-α, IKK-β, and IKK-γ.* ***2****. Phosphorylation of inactive IκBα (the inhibitory unit of the NF-kB (p65/p50) complex).* ***3****. Dissociation and degradation of IκBα to release the active NF-κB p50/p65 heterodimer.* ***4****. Translocation of NF-κB to the nucleus.* ***5****. Binding of NF-κB and DNA to activate inflammatory, degradative and apoptotic gene production (Roman-Blas and Jimenez, 2006; Shakibaei et al., 2007; Csaki et al., 2009). Inhibitory effects of various plants extracts have been measured at these stages (indicated by a blue line). A = avocado/soybean unsaponifiables, C = curcumin, E = epigallocatechin gallate, N = nobiletin, P = pomegranate extract, R = resveratrol.*

Some of these extracts have been tested in horses and were found to have disease-modifying properties. For example, oral ASU treatment of horses with experimentally induced OA significantly reduced the severity of articular cartilage erosion and synovial haemorrhage whilst significantly increasing GAG synthesis, compared with placebo-treated horses, although it had no effect on signs of pain or lameness (Kawcak *et al.*, 2007). Therefore, there are many known plant-derived extracts with potential joint supportive properties, and probably many more as yet undiscovered. However, their modes of actions and beneficial effects must be established in *in vitro* and *in vivo* models of OA before the widespread acceptance of the scientific community.

1.8. Current Models of Osteoarthritis

1.8.i. *In Vivo* Models

Pain in OA only manifests after the involvement of other joint structures with a nerve supply such as the synovium (Brandt, 1989), thus cartilage degeneration is often advanced before it is clinically diagnosed. Therefore, animal models have been developed to study OA at earlier stages and monitor disease progression. Many animal species are currently used in OA research including; laboratory animals: mice, rats, guinea pigs, rabbits; farm animals: sheep, goats; and companion animals: dogs, cats, horses. Animal OA models roughly fall into five categories; firstly, spontaneous OA, which naturally occurs in the knee joints of animals, such as guinea pigs and dogs, and has a similar pathogenesis to human OA (Miller and Lust, 1979; Bendele and Hulman, 1988). Secondly, the surgical creation of joint instability, for example anterior cruciate ligament transection (ACLT) in dogs (Pond and Nuki,

1973), meniscal tear model in rats (Janusz *et al.*, 2002), and collateral ligament transection in horses (Simmons *et al.*, 1999). Thirdly, the surgical replication of joint trauma, for example the canine groove model (Marijnissen *et al.*, 2002), and carpal chip fragmentation in horses (Kawcak *et al.*, 2008). Fourthly, injection into the joint, for example papain (Bentley, 1971), sodium mono-iodoacetate (Gustafson *et al.*, 1992) and collagenase (van der Kraan *et al.*, 1990). The final category is the knock-out model which deletes certain genes in mice resulting in the development of OA-like degenerative joint disease. For example the deletion of the gene that codes type IX collagen (Fässler *et al.*, 1994), or the double deletion of biglycan and fibromodulin (Ameye *et al.*, 2002). Some commonly used animal models of OA are summarised in figure 8.

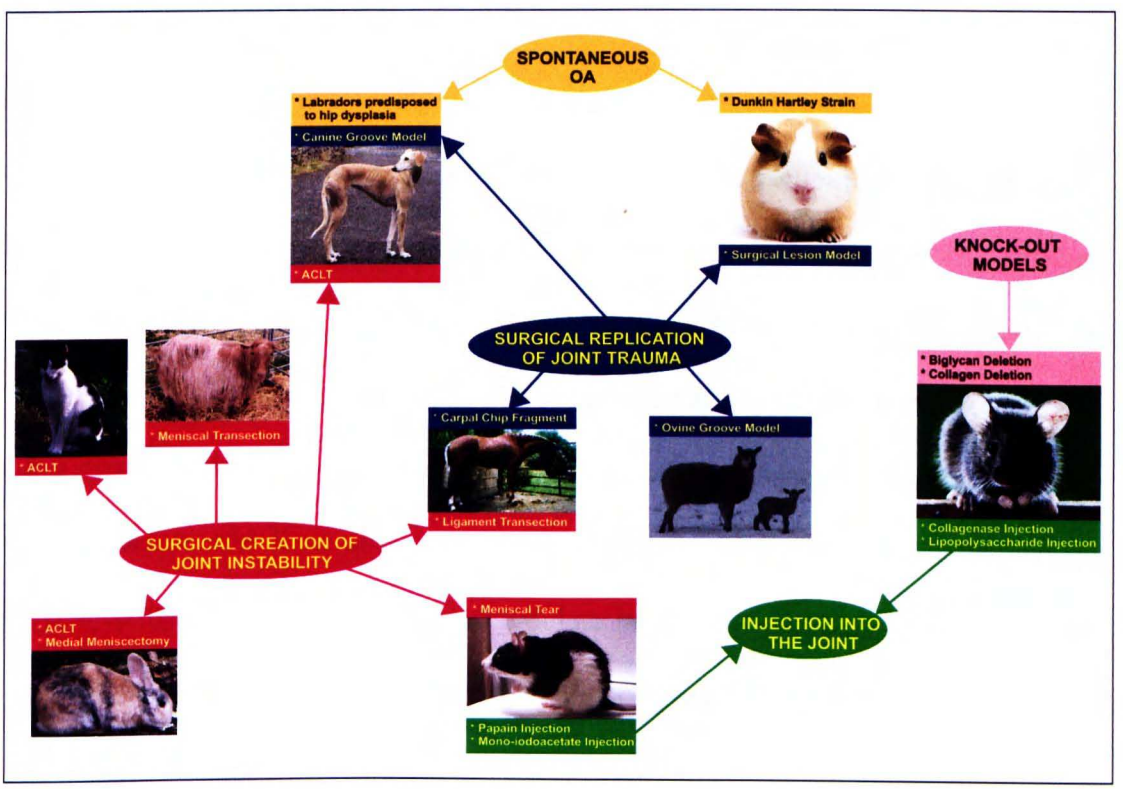

Figure 8. Commonly used animal models of osteoarthritis

ACLT = anterior cruciate ligament transaction; OA = osteoarthritis.

All these models cause different characteristics of OA and differ in their similarities with the human condition. For example, aggrecan content of cartilage from dogs with spontaneous OA decreased by 50-40% (similar to human OA) whereas it increased by 26% in cartilage from ACLT-induced OA (Liu *et al.*, 2003). In addition, some models such as the partial medial meniscectomised guinea pig model, produce rapid and severe cartilage degeneration which may limit their use for testing therapeutic agents (Bendele, 1987). The merits and limitations of each model must therefore be carefully considered when designing experiments and translating the results. In addition, there are obvious welfare issues with using live animal models in terms of pain, debilitation and euthanasia. Therefore, the development of alternative *in vitro* models of OA have been encouraged to reduce, refine and replace the use of animals in research.

1.8.ii. *In Vitro* Models

All *in vitro* culture systems are simplified models of an *in vivo* milieu (Stewart *et al.*, 2000). *In vitro* models, are often used as a preliminary step before *in vivo* testing, especially in determining safe concentrations of test substances (Pearson, 1986). *In vitro* testing has numerous benefits in that it can standardise experimental conditions, reduce costs, increase sample numbers, lessen variability through the elimination of systemic factors and limit animal suffering (Finn and Giardino, 2003). However, metabolites and toxic products can accumulate in *in vitro* cultures, and the relative simplicity of *in vitro* systems compared to the complex interactions occurring in an organism, limits their use (Finn and Giardino, 2003).

With regard to *in vitro* models of cartilage and OA, there are numerous models available. Isolated chondrocytes can be grown in high density, stationary monolayers or in roller bottles where they form monolayers and multilayers, to examine morphology and responses to external stimuli at a cellular level (Kuettner *et al.*, 1982). However, this system is very different from the *in vivo* situation, where chondrocytes reside either singly or in pairs throughout the cartilage matrix. In the monolayer system chondrocytes can lose their phenotypic stability after several weeks, as they express more type I collagen and less collagen type II (Benya *et al.*, 1978). In addition, the morphology of monolayer chondrocytes changes from spherical to flattened, fibroblast-like cells after about a week in culture (Grundmann *et al.*, 1980). Consequently, more appropriate scaffolds for chondrocyte culture have been investigated, notably on alginate beads or within agarose gels.

Chondrocytes grown in alginate beads (either in a semi-solid gel or encapsulated within hollow beads) maintain their spherical morphology as opposed to the typical fibroblast-like morphology of monolayer chondrocytes (Guo *et al.*, 1989). Chondrocytes that have de-differentiated in monolayer culture will re-differentiate back to their chondrocyte phenotype after culturing on alginate beads for two weeks (Lemare *et al.*, 1998) or after a week in agarose gels (Benya and Shaffer, 1982). In addition, adult bovine articular chondrocytes remain phenotypically stable for up to eight months when grown on alginate beads, expressing collagen type II and aggrecan (Hauselmann *et al.*, 1994). Chondrocytes grown in monolayers lose their responsiveness to IL-1β over time, for example reduced NO production that is non-detectable by passage six (Blanco *et al.*, 1995), and reduced MMP-9 production by passage two, which can be restored by culture in alginate (Lemare *et al.*, 1998). Interestingly, O_2 tension is also thought to be important for restoring and maintaining

the chondrocyte phenotype alongside alginate. Although the aforementioned studies showed re-differentiation of chondrocytes in alginate beads under standard culture conditions, other studies have shown re-differentiation only when cultured in alginate at low O_2 tension (5%), as opposed to standard normoxic levels (20-21% O_2) (Murphy and Sambanis, 2001; Domm *et al.*, 2002). Thus, it appears low O_2 and a three-dimensional (3-D) culture system are important for maintaining the chondrocyte phenotype. Thus, alginate beads are useful for long-term chondrocyte culture studies, and are of value when studying chondrocyte responses to inflammatory mediators.

In contrast to isolated cell models, the cartilage explant model maintains the chondrocytes in their native matrix. Although chondrocytes in cartilage explants maintain their phenotypic stability, they have lower basal expressions of matrix proteins than are found *in vivo* (Stewart *et al.*, 2000). Despite this, explant models are often used to study the synthesis and turnover of ECM molecules (Carney *et al.*, 1985; Campbell *et al.*, 1989). The explant model has a variety of uses. It can use arthritic and non-arthritic cartilage from age matched controls for comparative studies on matrix composition and change (Malemud *et al.*, 1995). However, the often unknown aetiology of OA and individual variation, for example in weight and inflammatory response, can make comparisons between individuals difficult. Therefore, another adaptation of the model is to use healthy cartilage stimulated with various stimuli, alone or in combination, to induce changes typical of OA. This enables cartilage from the same donor to be used for controls and treatments alike, thereby reducing variation, and allowing the effects of different stimuli to be evaluated. Thus, explant models can provide more precise and scientifically robust

studies of cartilage in a constant environment, removing many of the confounding factors associated with live animal work.

The stimulus used to induce degradation in the explant model can determine the nature of OA being studied. For example, the addition of a single cytokine such as IL-1β to human cartilage explants increases gene expression of degradative enzymes and inflammatory mediators, whilst reducing collagen type II expression (Sandell *et al.*, 2008). Collagen type II gene expression is significantly lower in cartilage from early OA patients compared to late OA patients (Aigner *et al.*, 2006), thereby suggesting that the IL-1β-stimulated explant culture system represents a model of early OA.

Other explant systems have used combinations of cytokines to induce cartilage degradation, in terms of GAG and/ or collagen loss, at lower concentrations than each cytokine alone. This has been done with various combinations, such as IL-1β and TNF-α in bovine nasal explants (Saklatvala, 1986), IL-1β and OSM in feline articular cartilage (Gabriel *et al.*, 2010), IL-17 with either OSM, IL-1α or TNF-α in bovine nasal cartilage (Koshy *et al.*, 2002). In contrast to the relatively rapid loss of GAGs from explants in culture, collagen degradation is often observed after 2 weeks of explant culture with combined cytokines (Koshy *et al.*, 2002; Gabriel *et al.*, 2010). However, the combination of cytokines with the plasma serine protease, activated protein-C, has been shown to induce collagen loss within 4 days (Garvican *et al.*, 2010), thereby highlighting the flexibility of the model. Despite this, the aforementioned explant models do not take into account the biomechanical forces present in the joint *in vivo*. Therefore, mechanical compression can be applied to explants in culture to represent joint injury through overloading. Injurious compression to cartilage explants reduces the tensile strength of the collagen

network, increases chondrocyte death and causes a prolonged increase in proteoglycan turnover (Quinn *et al.*, 1998). It also stimulates inflammatory pathways by inducing COX-2 and mPGES-1 expression and protein synthesis (Gosset *et al.*, 2006). To create a more complex model studying the interactions between mechanical forces and degradative pathways, injured cartilage explants can be cultured with TNF-α and IL-1β to cause a synergistic increase in GAG loss (Patwari *et al.*, 2003).

Cartilage explants can be studied in conjunction with cell culture, or explants of other joint structures to examine the effects of different joint components. For example, co-culturing cartilage explants with synoviocytes has suggested that the synoviocytes exert a protective effect over IL-1β mediated cartilage degradation (Gregg *et al.*, 2006). Culturing cartilage explants with subchondral bone (either attached to the cartilage or as separate explants) has been shown to increase chondrocyte survival compared to cartilage alone (Amin *et al.*, 2009). Therefore, there are a variety of *in vitro* chondrocyte and cartilage explant culture models for studying healthy and naturally diseased joint structures, as well as inducing cartilage changes typical of OA in normal cartilage.

1.9. Aims and Objectives

The aim of this thesis was to develop *in vitro* models of equine cartilage to investigate the anti-inflammatory and anti-degradative effects of plant extracts in order to assess the potential of using nutritional intervention to support joint health in equine OA.

In order to achieve the specific aims and objectives of this project, it was necessary to establish and test *in vitro* models of equine cartilage using a series of downstream biochemical assays. The models included monolayer cultures of primary chondrocytes for cytotoxicity testing and refinement of the well-established explant system for biochemical assays. A significant amount of time and effort was involved in developing and evolving the models in order to achieve reliability and consistency. Once the models were developed, it created the opportunity to perform hypothesis driven research, which resulted in the experimental chapters. The model needed to be validated and therefore the well-studied anti-inflammatory phytochemical agent, curcumin, and the NSAID, carprofen, were employed to test the *in vitro* models before studying combination products developed by WALTHAM® and by third parties.

An unexpected benefit of the developmental phase of this study was the opportunity to exploit the refined explant model for proteomic studies of the cartilage secretome in collaboration with Bruker Daltonics in Coventry.

Although not stated in the original proposal, the opportunity arose to conduct an *in vivo* field based study at Redwings Horse Sanctuary in Norfolk to gain experience in the area of field trial work by evaluating the combination products tested *in vitro*. This highlighted the limitations and complexities of field trial work and emphasised the importance of not over-interpreting *in vitro* work.

One of the anticipated outcomes was that the results of the *in vivo* field trial and the proteomics work would help direct future research in the area of selection and testing new botanical extracts for supporting joint health.

CHAPTER 2. DEVELOPING A CARTILAGE MODEL OF EARLY OSTEOARTHRITIS FOR TESTING PLANT EXTRACTS

2.1. Introduction

The chondrocyte is a highly specialised cell that synthesises components of the cartilage ECM. Within the matrix, chondrocytes are sparsely distributed, making up less than 10% of the ECM tissue volume (Carney and Muir, 1988). *In vitro*, chondrocytes grown in low density monolayer cultures begin to de-differentiate into fibroblast-like cells and synthesise collagen type I instead of type II, thus changing their phenotype (Muller *et al.*, 1977; von der Mark *et al.*, 1977). Cartilage explant models allow the study of cartilage *in vitro* without causing this de-differentiation, and although the mRNA expression of cartilage matrix components; pro-collagen type II, aggrecan and fibronectin, are down-regulated, this is thought to be as a consequence of removing biomechanical stimuli (Stewart *et al.*, 2000). Explants support viable cells and can respond to the addition of exogenous inflammatory cytokines and/or mechanical compression by exhibiting a dose-dependent release of cartilage GAGs from the ECM (Ratcliffe *et al.*, 1986; Patwari *et al.*, 2003; DiMicco *et al.*, 2004). GAG depletion of cartilage occurs in OA, thus, by adding these exogenous stimuli alone or in combination, the cartilage explant model can be adapted to create *in vitro* models of OA.

Although many studies have examined explants taken from osteoarthritic patients (Shuckett and Malemud, 1988; Billinghurst *et al.*, 1997), the differing (and sometimes unknown) aetiologies, duration of disease and status of disease progression can vary widely between subjects. Cartilage cultured *in vitro* from

osteoarthritic patients is thought to be less vital, in terms of increased catabolism and decreased anabolism, compared to normal cartilage shortly after collection (Lafeber *et al.*, 1992). This suggests that the properties of the explant model will be greatly affected by the disease status of the tissue. However, the response of cartilage explants to exogenous stimuli *in vitro* enables the utilisation of cartilage from patients with no clinical signs of joint pathology. Thus, apparently "healthy" cartilage from multiple donors can be stimulated at the same time to enable comparisons of potential treatments at a similar disease stage. Although this may not be identical, it at least allows for some control on the variability that is inherent with clinical patient samples. In addition, the model enables the investigation of the effects of various stimuli, such as cytokines and mechanical compression (Patwari *et al.*, 2003), both alone and in combination, to determine their individual effects on normal cartilage, whereas the multi-factorial aetiology of OA can make comparisons between patients difficult. Consequently, the cartilage explant model is a well-established *in vitro* model for examining cartilage under various culture conditions.

The aim of the studies in this chapter was to develop an *in vitro* explant model, stimulated with an inflammatory mediator to mimic the structural changes in early OA for future studies testing plant extracts for potential anti-catabolic and anti-inflammatory effects. The pro-inflammatory cytokines IL-1β and TNF-α were tested in the model to induce cartilage degeneration indicative of OA, as they have been found in the synovial fluid of osteoarthritic equine joints (Morris *et al.*, 1990; Billinghurst *et al.*, 1995). Once the stimulus was determined, the anti-inflammatory phytochemical, curcumin, was used to test the model. A combination of colorimetric assays, immunoassays and western blotting were used to quantify various

degradation products, inflammatory mediators and catabolic enzymes in the explant culture medium in response to inflammatory stimuli and plant extracts.

Due to the developmental nature of this chapter, the general methods are described first, then each of the studies charting the chronological development of the model are described in turn with the results and improvements for each study to show how the model developed. This is followed by a general discussion of the chapter.

2.2. General Materials and Methods

Tissue Collection

Full depth, macroscopically normal articular cartilage samples as determined by observation of a smooth intact cartilage surface, clear viscous synovial fluid and a non-inflamed synovium, were obtained from weight-bearing regions of the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints (unless otherwise stated) of horses of mixed breed, age and sex. Cartilage shavings were aseptically harvested into low glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Thermo Fisher Scientific) containing 4% penicillin/streptomycin (Sigma-Aldrich) before washing in phosphate-buffered saline (PBS) containing 10% penicillin/streptomycin for 20 minutes. Cartilage samples from each animal were kept separate throughout the studies.

Cartilage Histology

Histological samples of articular cartilage were taken from each joint to ensure that macroscopic identification of a healthy joint was supported by microscopic evidence. Cartilage was fixed in neutral buffered formalin for a minimum of 24 hours. Fixed

samples were processed overnight and embedded in paraffin. The blocks were sectioned at 5μm, mounted onto non-coated glass slides and left to dry. Tissue sections were then subjected to haematoxylin and eosin (H & E) staining. In brief, sections were de-waxed in Histo-Clear (National Diagnostics) for 30 minutes, followed by rehydration in a graded series of ethanol baths (100%, 95%, and 70%). Slides were washed in distilled water and stained with haematoxylin for 4 minutes. Slides were then washed in distilled water, dipped in 1% industrial methylated spirit (IMS), washed in distilled water, dipped in ammoniated water and washed in distilled water. Tissue sections were then counterstained with eosin for 5 minutes. After thorough washing with distilled water, the sections were dehydrated in another series of graded ethanol baths (70%, 95%, and 100%). Sections were cleared in Histo-Clear, then Xylene (Thermo Fisher Scientific) for 5 minutes before mounting in DPX (Fluka) and leaving to set. Stained sections were photographed at X200 using an inverted microscope (Leica DM5000B) fitted with a digital camera (Leica DFC350FX) and Leica Application Suite software. Sections were examined for irregularities in the articular surface.

Some examples of joints and histology are shown in figure 9. A joint designated as suitable for collection via macroscopic and histological evidence are shown in figure 9.A, whereas cartilage not suitable are shown in figure 9.B.

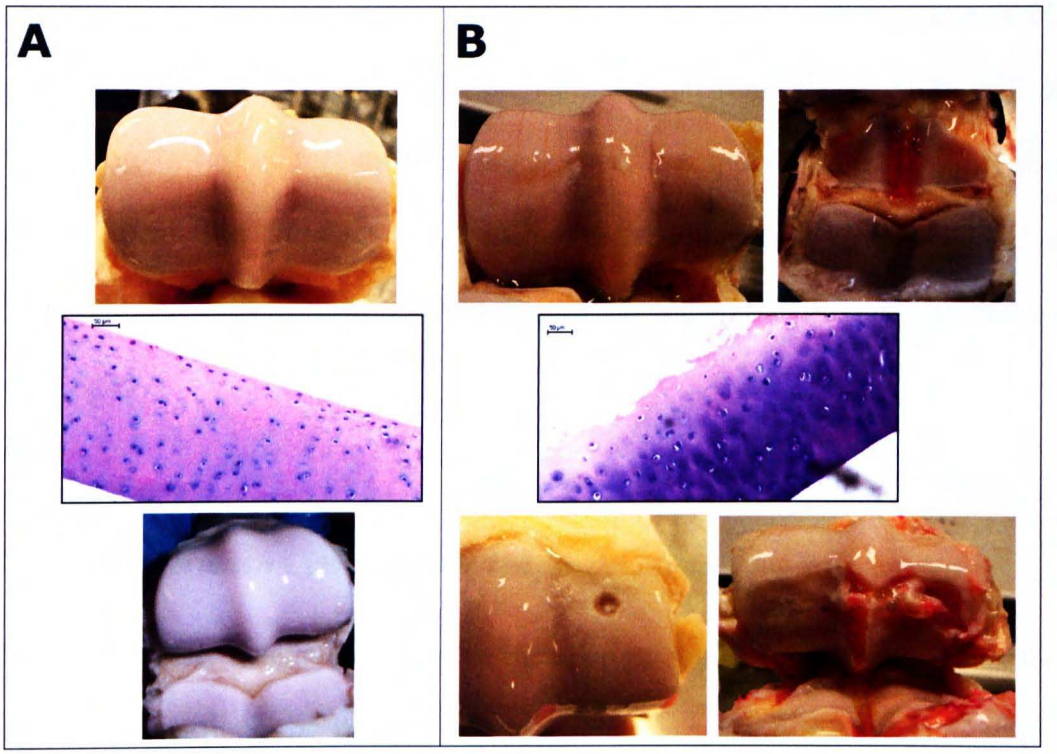

Figure 9. Selection of metacarpophalangeal joint cartilage for the explant model

A. *Photographs and histology slide of healthy cartilage as determined by observation of a smooth intact cartilage surface, clear viscous synovial fluid and a non-inflamed synovium. B. Photographs of unsuitable joints and haematoxylin and eosin stained tissue section of damaged articular cartilage. Criteria for exclusion included wear lines, fissures or irregularities on the articular surface, red synovial fluid, and/ or subchondral bone cysts.*

Cartilage Explant Preparation

For all studies, full depth cartilage shavings were cut into 3mm discs using a biopsy punch. Three discs per well from the same animal were placed in 24-well plates containing 1ml of culture medium (serum-free, low glucose DMEM supplemented with 2% penicillin/streptomycin) and allowed to equilibrate overnight at 37°C under 5% CO_2. The following day, before the studies began, the overnight culture media were removed.

2.3. Study 1. Human Cytokine Selection for the Model

This initial study sought to determine the appropriate inflammatory stimulus for the model as determined by the GAG assay, which quantitates the levels of sulphated GAGs in the media and explants. The study was carried out using human recombinant cytokines: IL-1β and TNF-α, due to their known ability to induce cartilage degradation through proteoglycan breakdown (Pratta *et al.*, 1989).

2.3.1. Materials and Methods

Cartilage was collected from the MCP and MTP joints of one horse. Explants were prepared as previously described.

Study Design

Overnight culture medium was removed and replaced with fresh culture medium containing the treatments (1ml total volume per well). Treatments included; recombinant human IL-1β (Roche Diagnostics; 0.5ng/ml, 10ng/ml, and 25ng/ml),

recombinant TNF-α (Roche Diagnostics; 0.5ng/ml, 10ng/ml, and 25ng/ml), and a combination of IL-1β and TNF-α (10ng/ml each). Each treatment was repeated in triplicate. Explants were incubated for five days at 37°C and 5% CO_2, after which time, media and explants were placed in separate 1.5ml microcentrifuge tubes and stored in a freezer at -20°C until ready to assay.

Glycosaminoglycan Assay

For evaluation of matrix GAG release, cartilage discs were digested in 1ml papain buffer (0.1M sodium phosphate buffer, 5mM N-acetyl cysteine, 5mM Ethylenediaminetetraacetic acid (EDTA)) containing 1.5mg/ml papain (Sigma-Aldrich) for 16 hours. Papain-digested cartilage and their corresponding supernatants were assayed in 96-well plates using the dimethylmethylene blue (DMMB; Sigma-Aldrich) method as described by (Farndale *et al.*, 1982). The assay is colorimetric and quantifies sulphated GAGs in suspension through the dye molecules associating with the sulphate, causing a shift in absorption as the dye turns from blue to pink. In brief, each sample was diluted in duplicate in distilled water to a volume of 40µl per well. Although dilutions varied to ensure that the samples were read from the middle of the standard curve for accuracy, they were approximately 1:5 for media samples and 1:40 for digested cartilage. Shark chondroitin sulphate (Sigma-Aldrich) in papain buffer was used to prepare standards (0 - 70µg/ml). DMMB solution was prepared (16mg 1,9-DMMB in 1 litre of water containing 3.04g glycine, 2.37g sodium chloride, 95ml 0.1M hydrochloric acid, pH3) and added to each well (200µl/well). Plates were immediately read on a plate reader (Multiskan Ascent, Thermo LabSystems) and assayed using Ascent Software (version 2.6, Thermo LabSystems). Total GAG release was obtained from a spectrophotometric

reading of the digested cartilage and its corresponding supernatant at 540nm. Percentage of GAG release from the total GAG content of the explants was calculated by dividing the supernatant value from the total GAG release for each well.

Statistical Analysis

Percentage GAG release data from each treatment in triplicate were combined and a one-way between-groups ANOVA was conducted to test for a statistically significant difference at the $p<0.05$ level. *Post hoc* comparisons using the Tukey test were conducted with GraphPad Instat (version 3.05, GraphPad Software Inc.) to determine where the differences among the groups occurred. Data were plotted using GraphPad Prism (version 4, GraphPad Software Inc.). Values are reported as means of three replicates per treatment from one horse ± standard error of the means (SEM).

2.3.2. Results

Control explants released a mean percentage of 11.92 ± 0.82% GAG over 5 days (figure 10). Recombinant human IL-1β significantly increased GAG release compared to controls at 0.5ng/ml (29.18 ± 3.79%, $p<0.001$), 10ng/ml (32.30 ± 2.58%, $p<0.001$) and 25ng/ml (31.55 ± 0.69%, $p<0.001$). TNF-α significantly increased GAG release, compared to controls, at 25ng/ml (22.78 ± 2.07%, $p<0.05$). IL-1β and TNF-α combined (10ng/ml each) resulted in a mean GAG release of 29.77 ± 3.23%, which was significantly higher than controls ($p<0.001$).

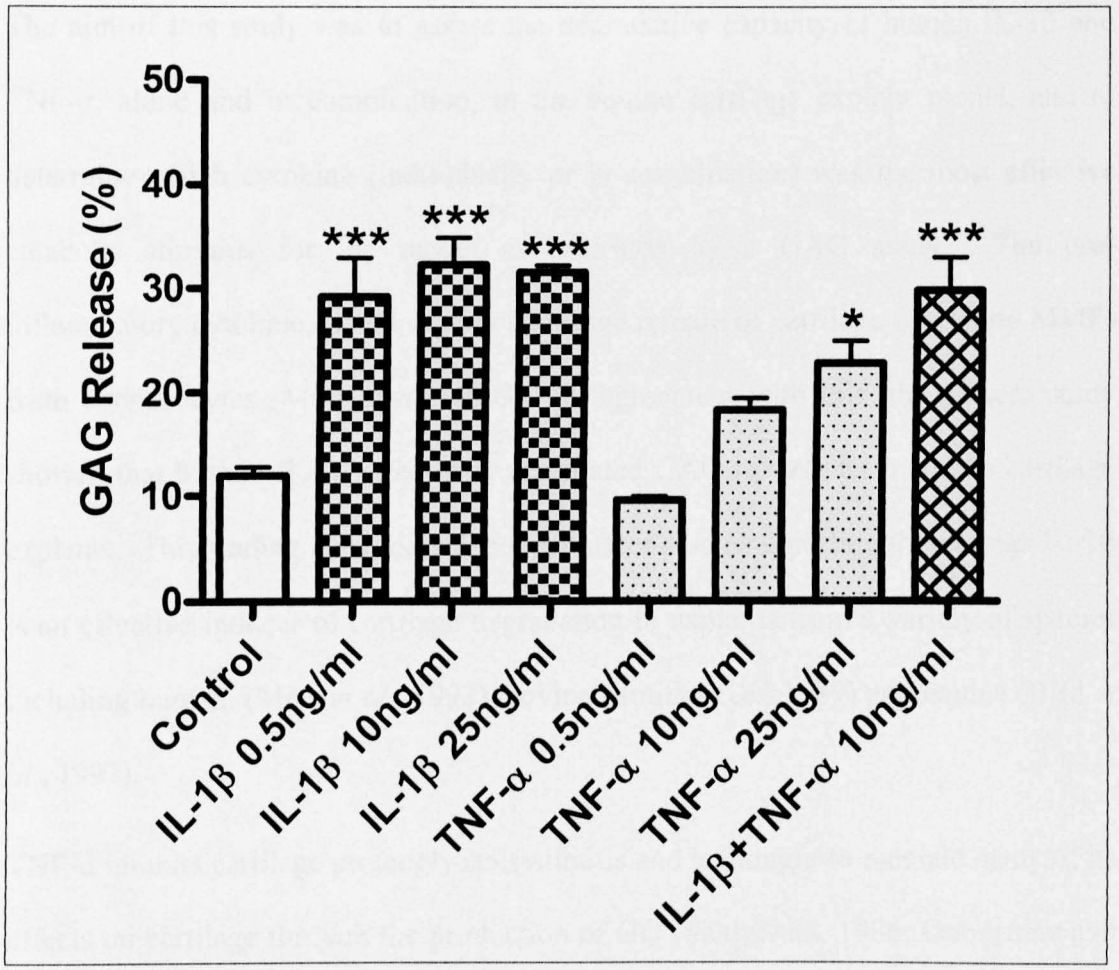

Figure 10. Percentage of glycosaminoglycans (GAGs) released from cartilage explants after 5 days, in response to recombinant human interleukin-1beta (IL-1β), tumour necrosis factor-alpha (TNF-α), or in combination

*Graphs give mean values collated from replicate wells (3 replicates per treatment) and bars represent standard error of the means (SEM). Significance compared to control explants is shown by * ($p<0.05$) and *** ($p<0.001$).*

2.3.3. Discussion

The aim of this study was to assess the degradative capacity of human IL-1β and TNF-α, alone and in combination, in the equine cartilage explant model, and to determine which cytokine (individually or in combination) was the most effective catabolic stimulus for the model as indicated by a GAG assay. The pro-inflammatory cytokine, IL-1β, causes increased release of cartilage degrading MMPs from chondrocytes (Mort *et al.*, 1993). In agreement with this, the present study showed that human IL-1β effectively stimulated GAG release from equine cartilage explants. This finding concurs with several other studies reporting that human IL-1β is an effective inducer of cartilage degradation in explants from a variety of species including human, (Mort *et al.*, 1993), bovine (Smith *et al.*, 1989) and equine (Bird *et al.*, 1997).

TNF-α inhibits cartilage proteoglycan synthesis and is thought to mediate many of its effects on cartilage through the production of NO (Saklatvala, 1986; Goodstone and Hardingham, 2002). As well as reducing cartilage anabolism, TNF-α also has catabolic effects, being capable of inducing focal loss of cartilage in OA (Westacott *et al.*, 2000). In the cartilage explant model, human TNF-α produced a significant increase in GAG release from the explants at a concentration of 25ng/ml. This is lower than some previous reports in other species where increased GAG release compared to control was not seen until seen at 100ng/ml TNF-α in bovine MCP cartilage explants (Gilbert *et al.*, 2004). However, other studies have found concentrations as low as 10ng/ml TNF-α can increase proteoglycan degradation in cartilage explants (Pratta *et al.*, 1989) and meniscal explants (Voigt *et al.*, 2009). The high variation in levels of TNF-α receptors within joints and between individuals (Westacott *et al.*, 2000), may account for differences between studies. This may also

explain the lack of significance at concentrations under 25ng/ml seen in the present study. Thus, further studies with larger numbers of animals may be needed to account for the individual variation in response to TNF-α.

Due to the complex nature of OA, it was decided that a combination of the two cytokines should also be investigated. The involvement of the two cytokines in OA is evident from the combined use of receptor antagonists to IL-1β and TNF-α to reduce collagen type II cleavage and GAG release from human OA cartilage explants (Kobayashi *et al.*, 2005). The combination of IL-1β and TNF-α (10ng/ml each) significantly increased GAG release compared to control. Combining cytokines has been shown to have a greater effect on GAG release than either cytokine alone. For example, in bovine nasal explants, TNF-α alone (2ng/ml) caused approximately 30% GAG release, IL-17 alone (0.5ng/ml) caused approximately 35% GAG release, whereas a combination of the two (2ng/ml + 0.5ng/ml) combined caused approximately 65% GAG release (Koshy *et al.*, 2002). However, the current study showed that, in terms of GAG release, there was no advantage of using two cytokines in the model. Therefore, in the interests of economy, IL-1β alone was selected for the model as it effectively increased GAG release at all the concentrations tested.

However, this study only provided preliminary results from one horse; therefore, a further study was required with larger numbers of animals to determine the reliability of IL-1β to consistently increase GAG release from explants.

2.4. Study 2. Determination of Optimal Human IL-1β Dose Ranges

The initial study established that IL-1β was an effective inducer of cartilage degradation in the model as indicated by the GAG assay. Thus, IL-1β was selected for the model, but to optimize the stimulus and check that results were consistent with the previous study, cartilage explants from multiple horses were subjected to a range of human recombinant IL-1β concentrations.

2.4.1. Materials and Methods

Cartilage from eight horses was used for the study. Each treatment was repeated in triplicate per horse, but not all horses were used for all treatments due to space constrictions on the plate, which resulted in different *n* numbers (where *n* = individual horses) for the various treatments. Control wells (n=8) contained 1ml of DMEM supplemented with 2% penicillin/streptomycin. Recombinant human IL-1β was diluted in the control medium to the following concentrations and added to each of the three wells per horse; 0.5ng/ml (n=4), 10ng/ml (n=5), 17ng/ml (n=3), 25ng/ml (n=4). Explants were then placed in an incubator at 37°C and 5% CO_2 for five days. Culture supernatants were placed into individual microcentrifuge tubes and stored in a freezer at -20°C before undergoing a GAG assay as previously described (page 53).

Statistical Analysis

Percentage GAG release data from each treatment in triplicate were combined for each horse and a one-way between-groups ANOVA was conducted to test for a statistically significant difference at the $p<0.05$ level. Data from all horses, except horse G which did not respond to IL-1β treatment, were then combined and analysed

with a one-way ANOVA. *Post hoc* comparisons using the Tukey test were conducted to determine where the differences among the treatment groups occurred. Values are reported as means of three replicates per treatment from seven horses ± SEM.

2.4.2. Results

Upon analysing the results from individual horses, it could be seen that cartilage from one horse (horse G) did not respond to the addition of IL-1β (figure 11). It was decided that data from this animal should be excluded from the analysis as it was a non-responder and would skew the data when combined with the other horses.

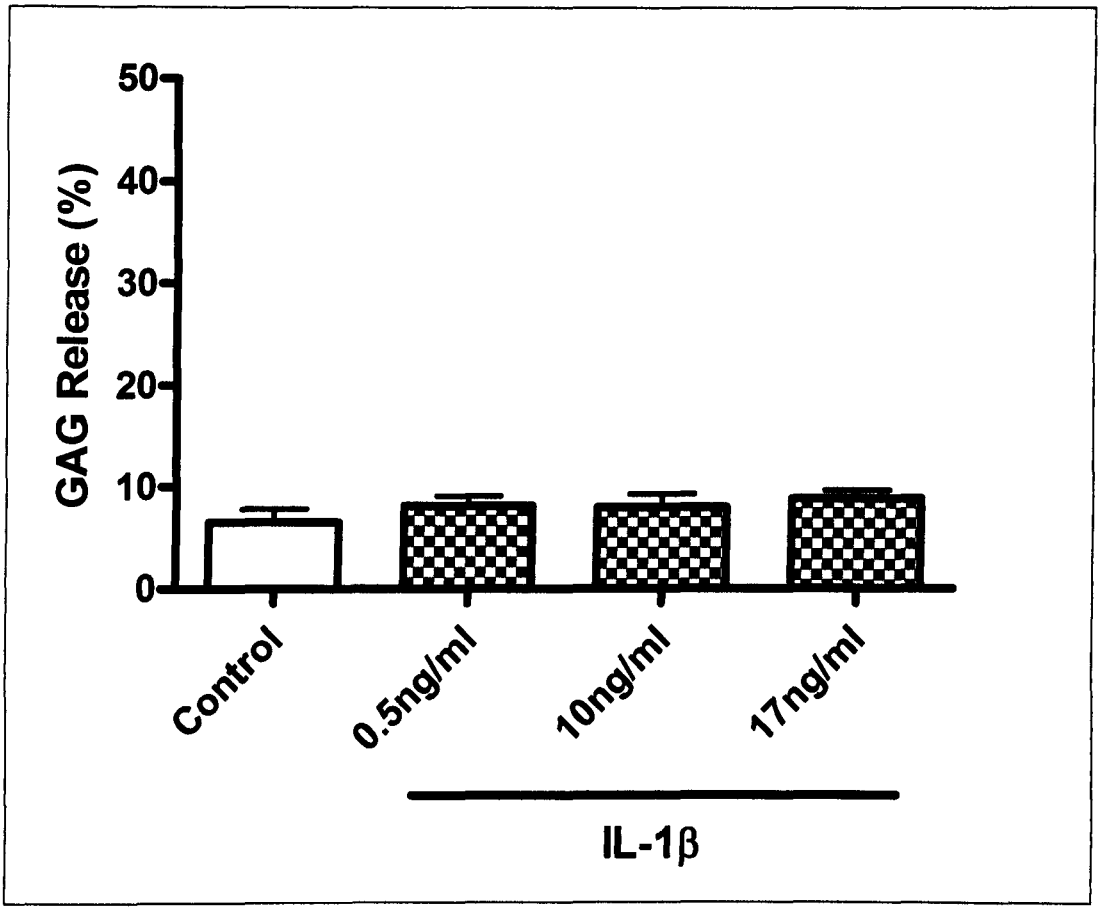

Figure 11. Percentage of glycosaminoglycans (GAGs) released from recombinant human interleukin-1beta (IL-1β)-stimulated cartilage explants from a non-responder (horse G) after 5 days

Graphs give mean values collated from replicate wells (3 replicates per treatment) and bars represent standard error of the means (SEM). No significant differences between control and IL-1β-treated explants were found.

Due to the omission of horse G from the results, the final number of horses included was n=7, although not all horses received all doses of the cytokine due to space restrictions on the plates. At all the concentrations tested, IL-1β significantly increased GAG release compared to controls (figure 12).

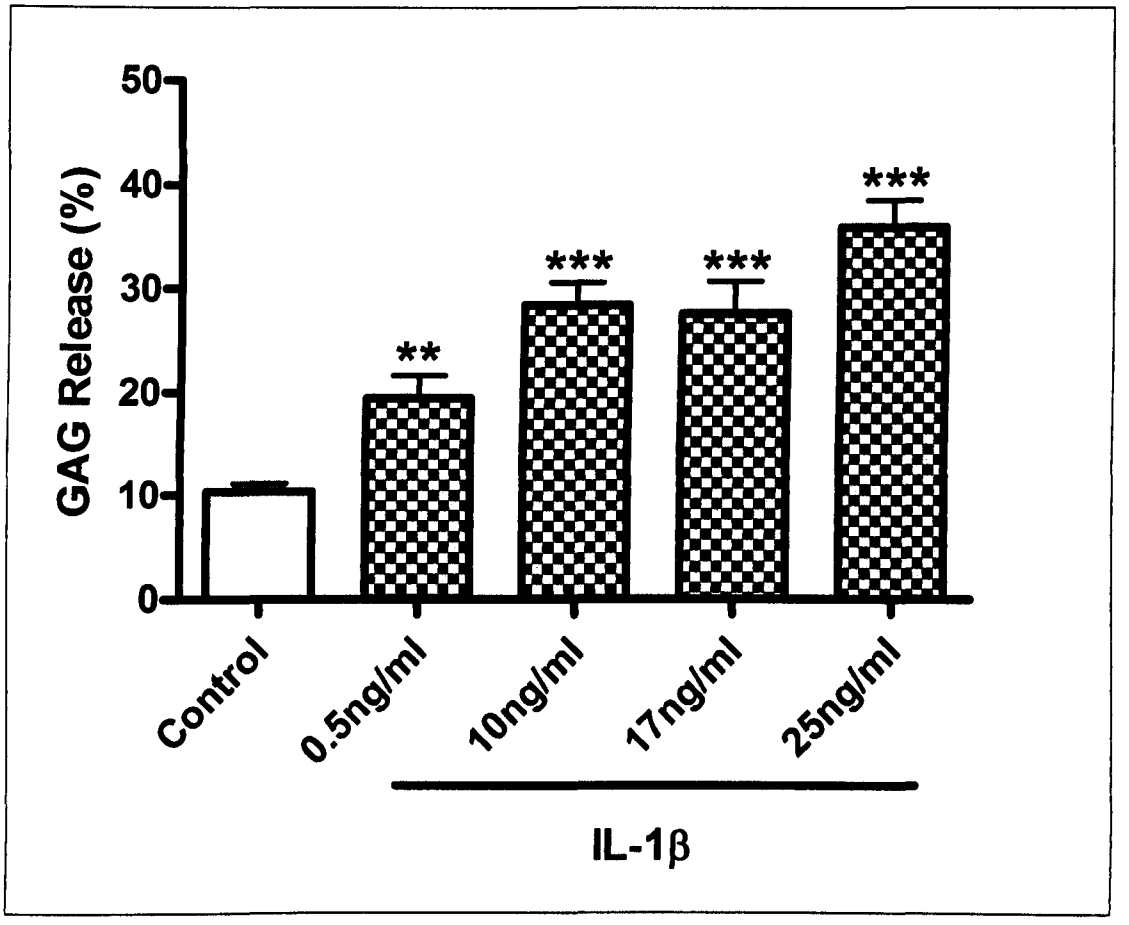

Figure 12. Percentage of glycosaminoglycans (GAGs) released from cartilage explants after 5 days, in response to recombinant human interleukin-1beta (IL-1β)

*Graphs give mean values collated from replicate wells (3 replicates per horse per treatment) and bars represent standard error of the means (SEM). Significance compared to control explants is shown by ** ($p<0.01$) and *** ($p<0.001$).*

Control explants from the seven horses released a mean percentage GAG release of 10.35 ± 0.80% over five days. GAG release was significantly increased from controls by the addition of recombinant human IL-1β at 0.5ng/ml (19.42 ± 2.23%, n=4, p<0.01), 10ng/ml (28.39 ± 2.22%, n=5, p<0.001), 17ng/ml (27.61 ± 3.08%, n=3, p<0.001) and 25ng/ml (35.93 ± 2.57%, n=4, p<0.001).

2.4.3. Discussion

In agreement with the previous study, recombinant human IL-1β significantly increased GAG release at all concentrations tested compared to controls in a larger number of horses. Thus, it was decided that IL-1β was a suitable stimulus for the model. With regard to consistency, the percentage GAG data in this study using multiple horses was within 5% of the values reported in the previous study using one horse for 10ng/ml and 25ng/ml IL-1β. However, there was larger variance of about 10% for 0.5ng/ml IL-1β, which affirms why it was important to test the variability in response to IL-1β with multiple horses. Similar to the previous study, IL-1β (10ng/ml) produced an increase in GAG release which was significant at the p<0.001 level. This concurs with several other studies that have used human IL-1β (10ng/ml) to significantly increase GAG loss in other species as measured by either increased content in medium or decreased GAG content in the cartilage itself (Venkatesan *et al.*, 2004; Miller *et al.*, 2007).

One horse was excluded from the results (horse G) as no response, in terms of GAG release, was observed with the addition of IL-1β. This could have been due to experimental error, but more likely the physiological status of the cartilage, notably age. Decreased responsiveness to cytokine stimulation has been noted in cartilage

from older horses (MacDonald *et al.*, 1992; Fuller *et al.*, 2001). The age of horse G was unknown and it is reasonable to suggest that age could have accounted for the lack of response to IL-1β treatment. The exclusion of horses that do not respond to catabolic stimuli has been reported previously (Petrov *et al.*, 2005). Therefore, it was decided that data from this animal should be excluded from the analysis as it was a non-responder and would skew the data when combined with the other horses. This set the precedent for future studies that if cartilage explants from a horse did not respond to IL-1β addition with a significant increase in GAG loss, the data from that animal should be excluded from the overall analysis.

2.5. Study 3. Testing the Human IL-1β-Stimulated Model Using Curcumin and IGF-1

The aim of the next study was to use the phytochemical curcumin to test the model as it has known anti-inflammatory properties, which may reduce cartilage degradation. Curcumin (diferuloylmethane) is a polyphenol found in turmeric derived from the rhizomes of *Curcuma longa*. Curcumin has both anti-inflammatory and anti-oxidant properties (Brouet and Ohshima, 1995; Sreejayan and Rao, 1997) and has been studied in clinical trials of patients with rheumatoid arthritis (RA) (Deodhar *et al.*, 1980). At concentrations between 50-100μM it has been shown to have anti-inflammatory properties via its suppressive effects on I kappa B kinase (IκB) activity and consequently the NF-κB signalling pathway in various cell types, including chondrocytes (Jobin *et al.*, 1999; Shakibaei *et al.*, 2007).

Curcumin is poorly soluble in aqueous solutions, but easily solubilised in organic solvents, such as dimethyl sulfoxide (DMSO) (Tonnesen and Karlsen, 1985). To

reduce any potential effects of DMSO on cartilage, a concentrated stock solution of curcumin (100mM) was prepared in DMSO to allow dilution in DMEM to a working concentration of no more than 0.01% DMSO in the highest curcumin concentration. Curcumin was tested against two IL-1β concentrations used in the previous study, 10ng/ml and 25ng/ml, to assess its effects on reducing GAG release on different parts of the dose response curve.

When testing the model with plant extracts it was important to include a positive control on each plate, to ensure that a reduction in GAG release was achievable. Recombinant human IGF-1 (10ng/ml) was selected as a positive control, due to its known antagonistic action on cytokine-induced proteoglycan release (Tyler, 1989).

2.5.1. Materials and Methods

Curcumin Preparation

Highly concentrated stock solutions (100mM) of curcumin extracted from *Curcuma longa* (Sigma-Aldrich, C1386) were prepared in DMSO (Sigma-Aldrich) and diluted down to a 1mM stock solution in DMEM, from which they were further diluted in the culture medium to their final working concentrations. In the highest concentration of curcumin tested in the model (100μM), the volume of DMSO did not exceed 0.01% of the 1ml total volume in each well. Multiple 100mM stocks of curcumin were aliquotted and frozen at -20°C.

Study Design

MCP and MTP joint cartilage from six horses was used for this study. A vial of 100mM curcumin stock (made as described above) was defrosted and diluted to a

1mM stock in DMEM. The following test concentrations were then further diluted in DMEM; 0.1μM, 0.5μM, 1μM, 10μM and 100μM. Each treatment used three wells on each of two 24-well plates per horse. The first plate for each horse contained the cartilage in culture medium with the extracts to assess the effect of curcumin on 'normal' cartilage. The second plate contained the cartilage in culture medium co-treated with curcumin and either 10ng/ml (n=3) or 25ng/ml (n=3) human recombinant IL-1β to induce cartilage inflammation and matrix degradation. These two concentrations were used so that the effect of curcumin could be assessed at different stages of the IL-1β dose response curve. Both plates contained culture medium, which acted as the control and the base medium for other treatments. Recombinant human IGF-1 (Roche Diagnostics; 10ng/ml) was included on the IL-1β-stimulated plates as a positive control, and on the non-IL-1β-treated plates to ensure it had no detrimental effects in the absence of IL-1β. Plates were incubated at 37°C and 5% CO_2 for five days. Supernatants and explants were placed into individual microcentrifuge tubes and stored in a freezer at -20°C for until undergoing a GAG assay as previously described (page 53).

Curcumin GAG Assay

Samples underwent a GAG assay as previously described (page 53), but first a preliminary test was undertaken to ensure that there was no native GAG present within the curcumin and/ or that the presence of curcumin did not change the colour of the dye thereby affecting the colorimetric reading of the assay. In order to do this, curcumin stocks (0.1μM, 0.5μM, 1μM, 5μM, 10μM, 100μM and 1mM) were prepared in water added to the wells of a 96-well plate in duplicate. The stocks were added to the wells undiluted (40μl per well) unlike when testing samples where they

are further diluted in water. This was to test the most concentrated curcumin samples for any effect. The DMMB solution was added and the plate was read as previously described (page 53). Photographs of the plate were taken as a visual addition to the results.

Statistical Analysis

Percentage GAG release data for each horse were statistically analysed individually. This highlighted that one horse in the IL-1β (10ng/ml) treated group was unresponsive to IL-1β, so it was removed from the combined analyses. Thus, all data from the non-IL-1β-treated samples in triplicate from five horses (not six) were combined and statistically analyzed using a one-way ANOVA with Tukey's multiple comparison *post hoc* test. Similarly data from the IL-1β treated plates were combined into either the 10ng/ml IL-1β treatment group (n=2) or the 25ng/ml IL-1β treatment group (n=3), with each group undergoing a one-way ANOVA with *post hoc* Tukey's test. For all analyses, statistical significance was set at $p<0.05$. Values are reported as means of combined animals + SEM.

2.5.2. Results

Curcumin contains no native GAG

To ascertain whether any native GAGs were present in the curcumin preparations that could affect the colorimetric reading of the assay, neat stocks of curcumin were tested from 0.1μM to 1mM in the 96 well plates (figure 13). The standards show the colour range of the DMMB assay ranging from pink (high levels of GAGs) to blue (low levels of GAGs). On the same plate, the curcumin stocks were assessed and it

could be seen from the blue colour that no GAGs were present. The GAG values obtained from this plate, showed that all concentrations of curcumin gave a value of 0μg sulphated GAG/ml from the assay, with the exception of 1mM which altered the colour of well and gave a value of 24.75μg/ml. However, the well was not pink in colour, which would have indicated the presence of GAG. Instead, the well was yellow, suggesting that the curcumin was affecting the reading, and not the presence of native GAG. It should be mentioned here that no explants were ever tested at this curcumin concentration (1mM). In addition, all the curcumin stocks were tested neat, whereas all treatment samples were heavily diluted in water before running on the plate. Therefore, it was concluded that at the concentrations tested, curcumin has no native GAG content which could affect the assay.

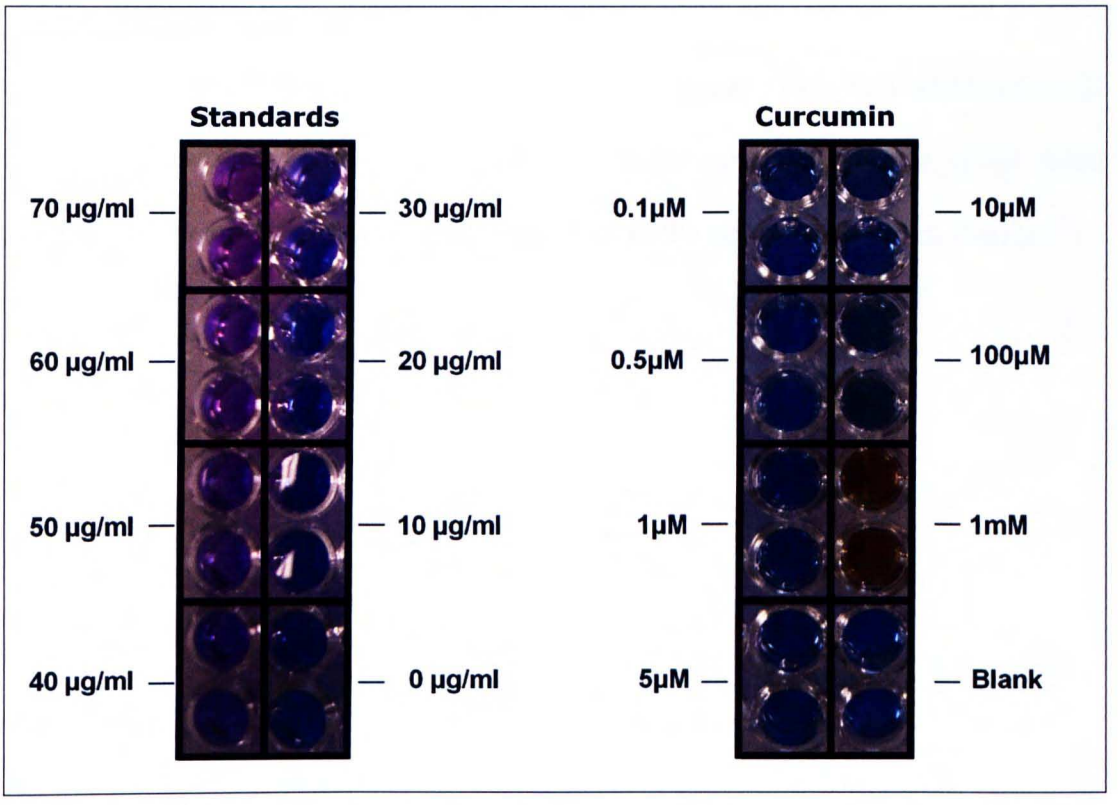

Figure 13. Annotated photographs of a glycosaminoglycan (GAG) assay plate

Plate contains the chondroitin sulphate standards (µg/ml) from which the standard curve is derived and the wells containing curcumin stocks. All wells contain the dimethylmethylene blue (DMMB) dye which turns pink when it reacts with sulphated GAGs.

Curcumin and IGF-I Do Not Significantly Change GAG Release From Unstimulated Cartilage Explants Compared to Controls

Control explants released a mean percentage GAG (mean GAG % ± SEM) of 11.02 ± 1.16% (figure 14). Neither IGF-1 nor any of the curcumin concentrations tested had any significant effects compared to controls in the non-IL-1β-treated samples.

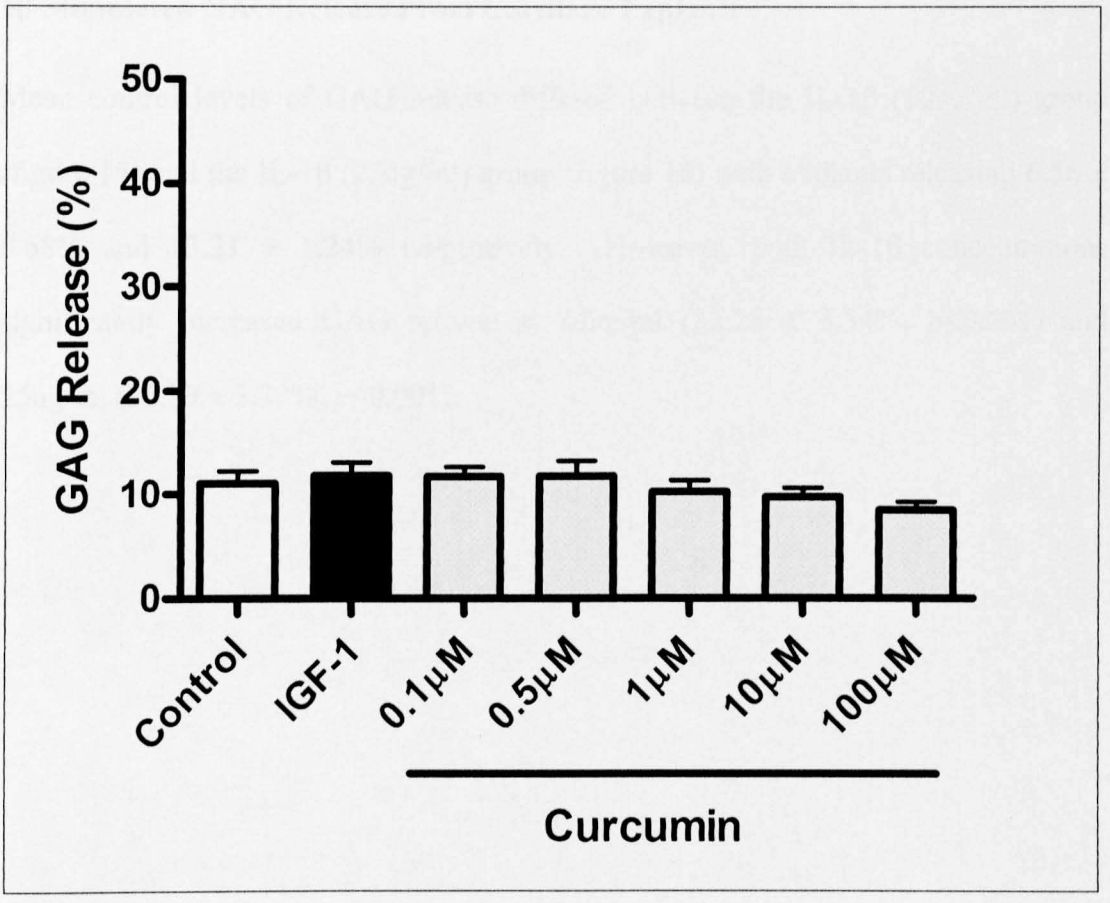

Figure 14. Percentage of glycosaminoglycans (GAGs) released from non-interleukin-1beta (IL-1β)-treated cartilage explants from five horses after 5 days, in response to insulin-like growth factor-1 (IGF-1; 10ng/ml) and curcumin (0.1-100μM)

Graphs give mean values collated from replicate wells (3 replicates per treatment) from 5 horses from 2 separate experiments. No significant differences compared to control were found. Bars represent standard error of the means (SEM).

Curcumin (100μM) and IGF-I (10ng/ml and 25ng/ml) Significantly Reduce IL-1β Stimulated GAG Release From Cartilage Explants

Mean control levels of GAG release differed between the IL-1β (10ng/ml) group (figure 15) and the IL-1β (25ng/ml) group (figure 16) with explants releasing 6.56 ± 0.68% and 13.21 ± 1.24% respectively. However, both IL-1β concentrations significantly increased GAG release at 10ng/ml (32.28 ± 5.34%, $p<0.001$) and 25ng/ml (37.39 ± 3.32%, $p<0.001$).

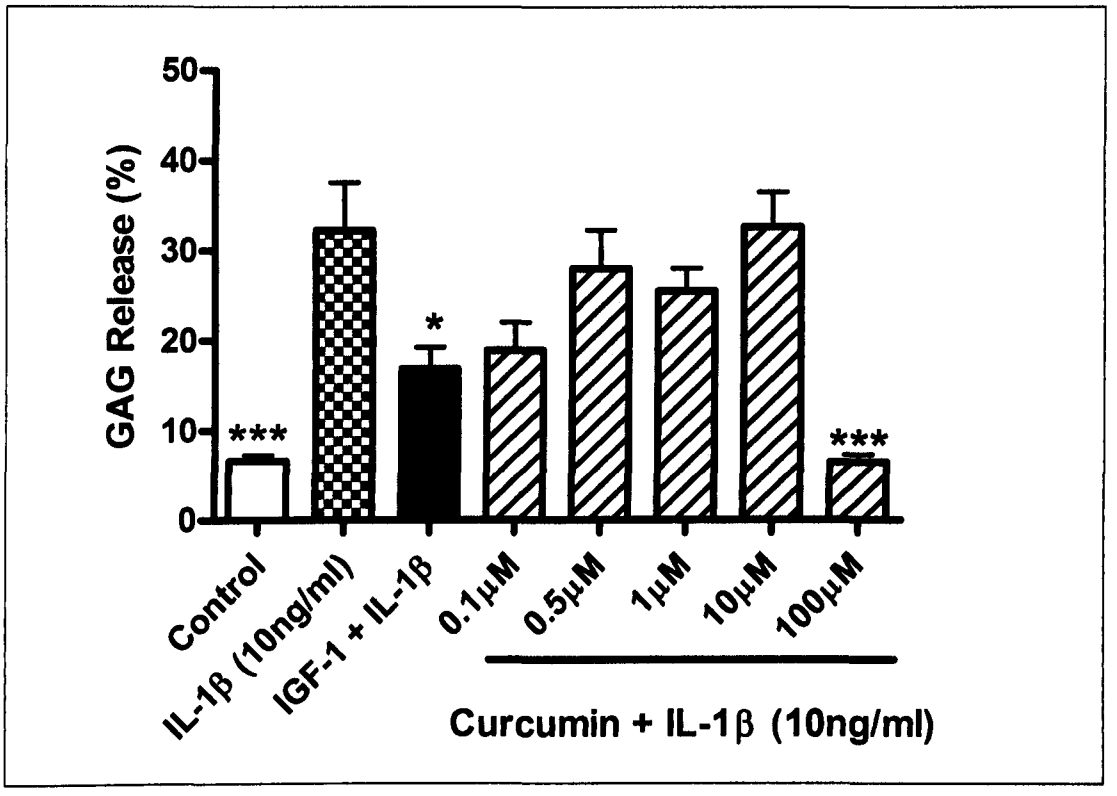

Figure 15. Percentage release of glycosaminoglycans (GAGs) into culture medium from cartilage explants treated with recombinant human interleukin-1beta (IL-1β; 10ng/ml) and insulin-like growth factor-1 (IGF-1; 10ng/ml) or curcumin (0.1-100μM)

*Significance compared to IL-1β is indicated by * ($p<0.05$) and *** ($p<0.001$). Graphs give mean values collated from replicate wells (3 replicates per treatment) from 2 horses and bars represent standard error of the means (SEM). Control indicates cartilage discs incubated in the culture medium alone.*

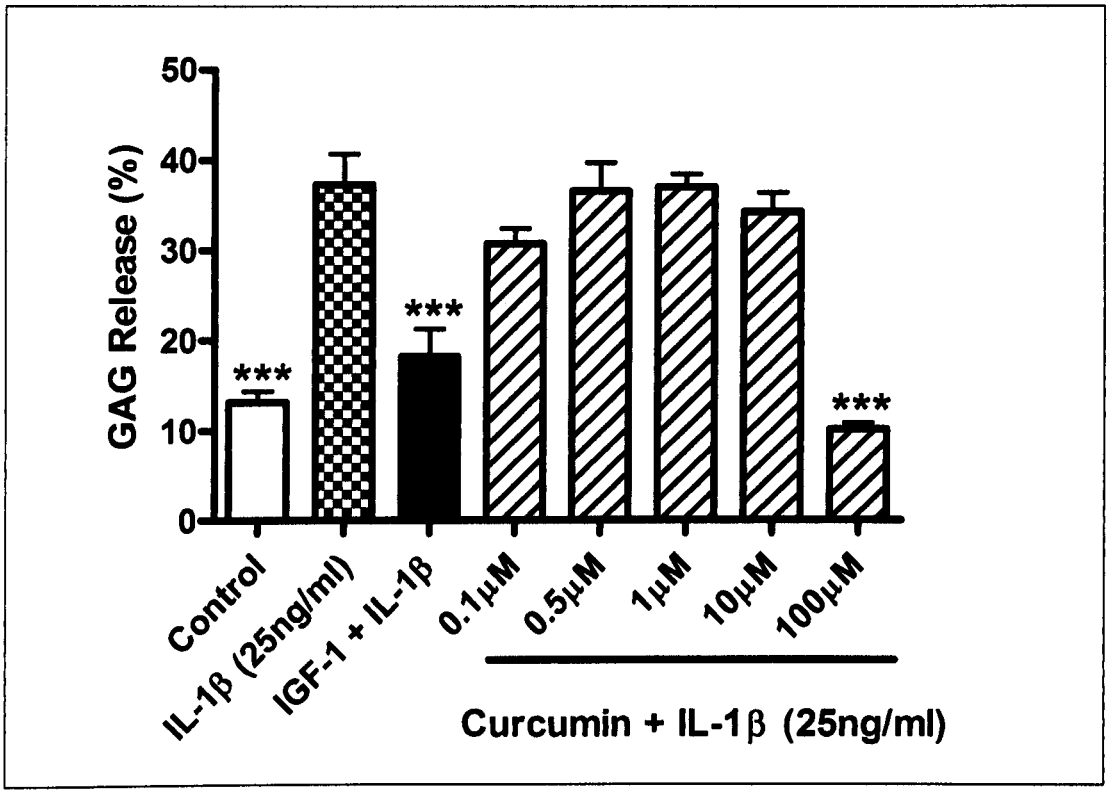

Figure 16. Percentage release of glycosaminoglycans (GAGs) into culture medium from cartilage explants treated with recombinant human interleukin-1beta (IL-1β; 25ng/ml) and insulin-like growth factor-1 (IGF-1; 10ng/ml) or curcumin (0.1-100μM)

*Significance compared to IL-1β is indicated by *** ($p<0.001$). Graphs give mean values collated from replicate wells (3 replicates per treatment) from 3 horses and bars represent standard error of the means (SEM). Control indicates cartilage discs incubated in the culture medium alone.*

IGF-1 (10ng/ml) significantly reduced IL-1β-stimulated GAG release at both 10ng/ml IL-1β (16.92 ± 2.34%, $p<0.05$) and 25ng/ml IL-1β (18.27 ± 3.03%, $p<0.001$). Curcumin, at concentrations between 0.1 and 10μM, had no significant effect on reducing GAG release from explants co-treated with IL-1β at either concentration. However, curcumin at 100μM significantly reduced the percentage of IL-1β-stimulated GAG release down to unstimulated control levels when exposed to IL-1β at either 10ng/ml (6.39 ± 0.84%, $p<0.001$) or 25ng/ml (10.16 ± 0.69%, $p<0.001$). This meant a reduction in GAG release from the explants by an average of 26% at 10ng/ml ($p<0.001$) and 27% at 25ng/ml ($p<0.001$).

2.5.3. Discussion

The aim of the present study was to use an *in vitro* explant model stimulated with an inflammatory mediator to induce cartilage changes typical of early OA and to test the model using curcumin, due to its anti-inflammatory effects and associated downstream benefits. Curcumin has been reported to protect human chondrocytes from IL-1β-induced activation and nuclear translocation of NF-κB thereby also protecting against inhibition of collagen type II and β1-integrin expression, and up-regulation of COX-2, MMP-9 and MMP-3 (Schulze-Tanzil *et al.*, 2004; Shakibaei *et al.*, 2007). Studies by other investigators have shown that curcumin inhibits MMP-1, MMP-3, MMP-13, ADAMTS-4 and TIMP-3 gene expression in chondrocytes stimulated with IL-1β, TNF-α and OSM, a member of the IL-6 superfamily of pro-inflammatory cytokines (Li *et al.*, 2001; Liacini *et al.*, 2002; Liacini *et al.*, 2003; Sylvester *et al.*, 2004). The results from the present study showed that curcumin at concentrations between 0.1μM and 100μM had no effect on unstimulated cartilage, suggesting that it has no gross effect on altering normal cartilage. However,

curcumin at 100μM effectively reduced IL-1β stimulated GAG loss down to control levels in the presence of both 10ng/ml and 25ng/ml IL-1β. The reduced GAG loss observed in the present study may be attributed to the inhibitory effect of curcumin on the NF-κB inflammatory pathway and the mediators of cartilage degradation such as MMPs and ADAMTSs. This effect could initially occur between the doses of 10μM and 100μM used in this study and further studies are warranted to determine whether the beneficial effect in reducing GAG release is seen within this concentration range. It is important to emphasize that the observation of reduced GAG release from the explants in the presence of higher concentrations of curcumin may not be a specific biological effect of curcumin. High concentrations of curcumin may, in fact, be toxic to chondrocytes (Toegel *et al.*, 2008). Therefore, any curcumin-induced cytotoxicity would interfere with proteoglycan metabolism in the explant model and this may be reflected in the results of the GAG assays. Consequently, the observed reduction in GAG loss from the explants in the presence of high concentrations of curcumin may be due to curcumin induced cell death, which would influence the expression of matrix degrading enzymes. Thus, chondrocyte viability testing will be an essential pre-requisite in future studies in order to determine the precise mode of action of curcumin in the explant model.

IGF-1 is a growth factor involved in cartilage anabolism and can counteract the degenerative effects of pro-inflammatory agents (Tyler, 1989). In this study, human IGF-1 (10ng/ml) effectively reduced IL-1β-stimulated GAG release without affecting cartilage GAG release in the absence of IL-1β. This is in agreement with previous studies which have found IGF-1 to counteract IL-1α or IL-1β-stimulated proteoglycan release from porcine (Fosang *et al.*, 1991b) and equine (Frisbie and Nixon, 1997) cartilage explants. Thus, IGF-1 was a suitable positive control for the

model. Although no effect on GAG release was observed with IGF-1 alone in comparison to controls, it does not mean it has no effect on unstimulated cartilage. It is more likely that the effects were not detected by the assay used in this study.

Although control levels of GAG release in individual animals varied, as would be expected with cartilage samples from individuals of unknown age, breed and background, the presence of IL-1β caused a significant increase in GAG release in all but one animal. The IL-1β-stimulated GAG release was always reduced to the control level for each individual animal by the addition of 100μM curcumin. Despite biological variation between animals, the reproducibility of the results suggests that this model is an effective *in vitro* system for testing the anti-catabolic potential of novel nutraceuticals and botanical extracts. These results suggest that in using curcumin, the GAG assay was able to pick up differences in GAG release consistent with the catabolic effects of IL-1β. It can be seen that curcumin antagonizes GAG release *in vitro*. However, further work is clearly needed to investigate the biological effects of curcumin at higher concentrations in order to determine its true efficacy and potential safety.

Despite the significant effects observed in this study suggesting that human IL-1β is a suitable catabolic agent in the model, several studies have raised concerns about the applicability of human IL-1β to experiments using cells from different species such as bovine and equine (Lederer and Czuprynski, 1989; May *et al.*, 1992). Using human recombinant IL-1β for high-throughput screening of anti-inflammatory botanicals, such as curcumin, that might support joint health in OA has distinct advantages over species specific IL-1β, in terms of lower cost and increased commercial availability. However, species-specific cytokines are more biologically relevant and hence, may be more appropriate for models studying the pathogenesis of

OA in a particular species. Therefore, it was decided that equine IL-1β should be investigated in the model.

2.6. Study 4. Using Equine IL-1β and Testing the Model Using Curcumin and IGF-1

In the interests of biological relevance, a second study was conducted using recombinant equine IL-1β in the model. Instead of repeating the previous experiments with multiple cytokines, equine IL-1β was selected as the cytokine of choice and was tested at 10ng/ml in conjunction with the previous studies and other published literature. Following on from previous work, the aim of the present study was to investigate the effects of equine IL-1β on cartilage degradation and inflammation, and to determine whether curcumin at concentrations between 10μM and 100μM could reduce these effects.

Due to the inflammatory nature of OA (Hedbom and Hauselmann, 2002) and the anti-inflammatory properties of many phytochemicals including curcumin (Jackson *et al.*, 2006), the level of the inflammatory mediating prostanoid, PGE_2, was measured in the media, in addition to the release of sulphated GAGs.

2.6.1. Materials and Methods

Study Design

MCP joints from three horses were used for this study to reduce variation. Explants were treated as either controls using culture medium alone or with recombinant equine IL-1β (R&D Systems; 10ng/ml) in culture medium, to a final volume of 1ml per well. A frozen vial of curcumin 100mM (made as previously described on page

65) was defrosted and diluted to 1mM in DMEM. This 1mM stock was diluted further in DMEM to form the test concentrations of 25μM, 50μM, 75μM and 100μM, which were added to wells to a total volume of 1ml containing IL-1β (10ng/ml) per well. Treatments were repeated in triplicate wells per horse. The study was conducted over two experiments due to supply issues with the abattoir. Two horses were assessed in one study, then repeated with another horse two months later.

The volume of DMSO did not exceed 0.01% of the 1ml per well in the highest concentration of curcumin tested. Explants were incubated for 5 days (37°C/ 5% CO_2), after which time both explants and corresponding supernatants were removed and frozen at -20°C until ready to assay for GAG content as previously described (page 53), or for PGE_2 content.

PGE_2 Assay (Normal Sensitivity)

A competitive immunoassay kit (R&D Systems) was used to measure PGE_2 according to the manufacturer's instructions. Standards (39 - 2050pg/ml) and supernatant samples (100μl) were added to a 96-well plate coated in goat anti-mouse polyclonal antibody, followed by 50μl primary mouse monoclonal antibody solution and 50μl of PGE_2 conjugate. The plate was covered and incubated for 2 hours at room temperature (21°C) on an orbital shaker at 500 revolutions per minute (rpm). The plate was thoroughly washed before adding 200μl substrate solution to each well and developing in the dark at room temperature for 30 minutes. Stop solution (50μl) was added and the plate was read immediately on a plate reader at 450nm with

wavelength correction set at 540nm using Ascent Software (version 2.6). The standard curve was calculated using a four parameter logistic curve-fit.

Statistical Analysis

Percentage GAG release data from the three horses were combined and statistically analyzed using a one-way ANOVA with Tukey's multiple comparison *post hoc* test. Similarly, PGE_2 data from the same samples were combined and analysed using a one-way ANOVA with *post hoc* Tukey's test. For all analyses, statistical significance was set at $p<0.05$. Values are reported as means of combined animals ± SEM.

2.6.2. Results

Curcumin Significantly Reduced IL-1β-Stimulated GAG Release (≥50μM) and PGE_2 Release (≥25μM)

Control explants from the three horses used for this study released a mean GAG percentage of 14.03 ± 1.36% after five days in culture (figure 17). The addition of IL-1β (10ng/ml) resulted in significantly higher GAG release from the explants (40.83 ± 3.21%, $p<0.001$). IGF-1 had no significant effect on reducing GAG release compared to IL-1β alone (36.00 ± 5.58%, n.s). Curcumin (25μM) did not significantly reduce IL-1β-stimulated GAG release (32.45 ± 7.85%, n.s). However, IL-1β-induced GAG release was significantly reduced by curcumin at 50μM (21.41 ± 2.89%; $p<0.05$), 75μM (15.93 ± 3.41%; $p<0.01$) and 100μM (9.78 ± 0.96%; $p<0.001$).

Explants in DMEM alone released 64.48 ± 8.93pg/ml (mean PGE_2 pg/ml ± SEM) PGE_2 over 5 days (figure 18). PGE_2 release was significantly increased by the presence of 10ng/ml IL-1β (226.40 ± 24.82pg/ml; $p<0.01$). IGF-1 did not significantly affect PGE_2 release compared to IL-1β alone (326.00 ± 66.97pg/ml; n.s). However, curcumin effectively attenuated the release of PGE_2 caused by IL-1β treatment at all concentrations tested; 25μM (54.65 ± 6.03pg/ml, $p<0.05$), 50μM (56.01 ± 9.18pg/ml, $p<0.01$), 75μM (75.40 ± 9.28pg/ml, $p<0.01$) and 100μM (64.98 ± 4.83pg/ml, $p<0.01$).

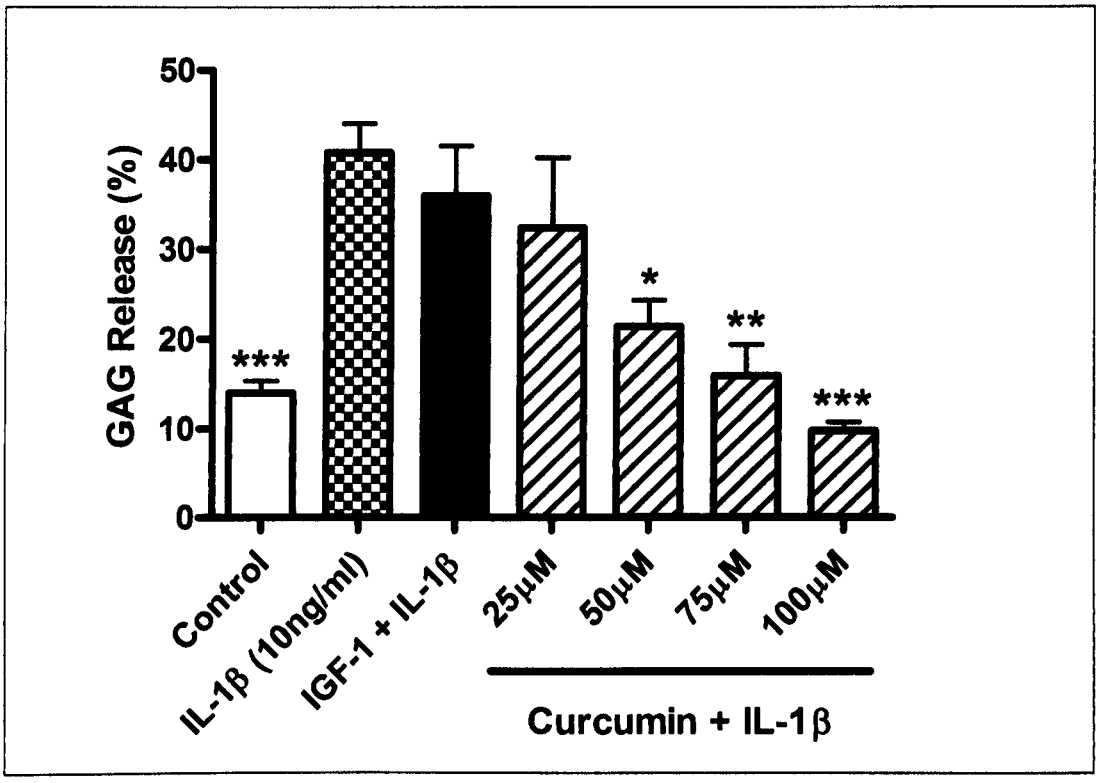

Figure 17. Percentage release of cartilage glycosaminoglycans (GAGs) into culture medium from recombinant equine interleukin-1beta (IL-1β; 10ng/ml)-treated cartilage explants in response to insulin-like growth factor-1 (IGF-1; 10ng/ml) or curcumin (25-100μM)

*Significance compared to IL-1β is indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). Graphs give mean values collated from replicate wells (3 replicates per treatment) from 3 horses and bars represent standard error of the means (SEM). Control indicates cartilage discs incubated in the culture medium alone.*

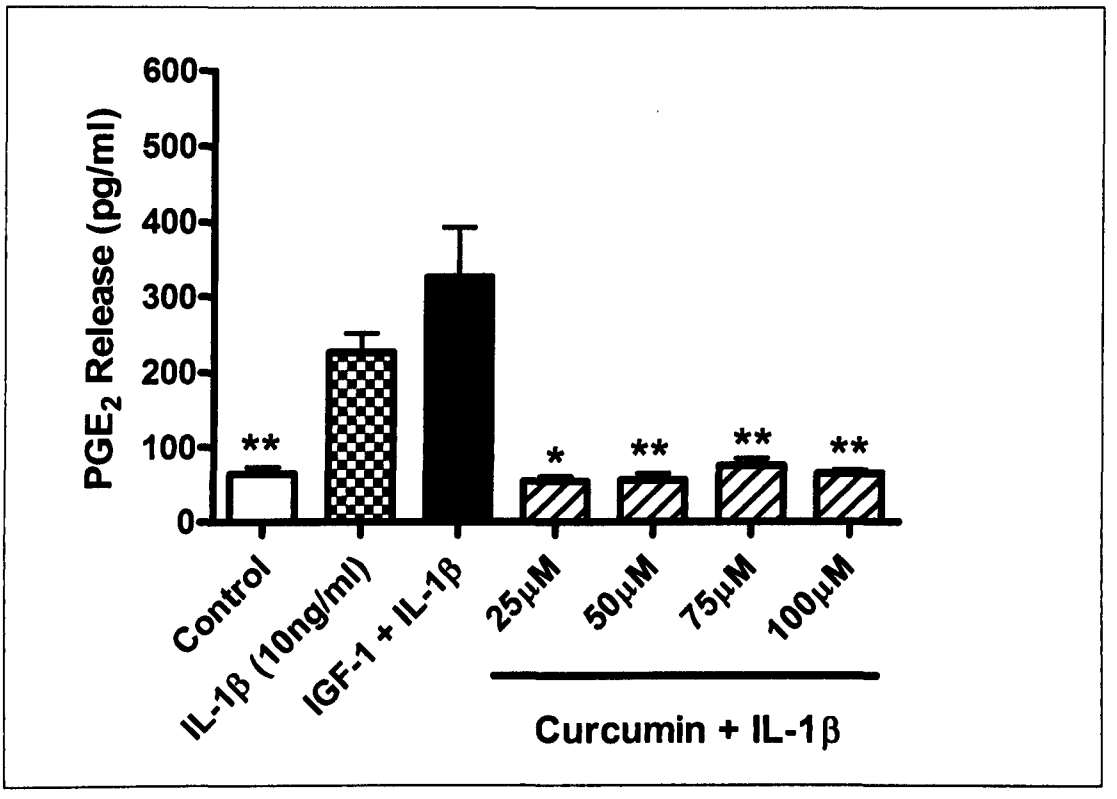

Figure 18. Prostaglandin E_2 (PGE_2) release from recombinant equine interleukin-1beta (IL-1β; 10ng/ml)-treated cartilage explants into culture medium in response to insulin-like growth factor-1 (IGF-1; 10ng/ml) or curcumin (25-100μM)

*Significance compared to IL-1β is indicated by * ($p<0.05$) and ** ($p<0.01$). Controls indicate cartilage discs incubated in culture medium alone. Graphs show the mean of 3 replicates from 3 horses per treatment and bars represent standard error of the means (SEM).*

2.6.3. Discussion

This study set out to ascertain whether equine IL-1β at 10ng/ml could increase GAG and PGE_2 release from cartilage explants in the culture media, and to determine whether curcumin could effectively reduce these species-specific cytokine effects.

Equine IL-1β (10ng/ml) significantly increased GAG release from the cartilage explants compared to controls. The mean GAG release from the equine IL-1β-treated explants was higher (40.83%), compared with the previous studies in the chapter using human IL-1β at the same concentration (32.30% study 1, 28.39% study 2 and 32.28% study 3). In terms of percentage increase from controls, the studies using human IL-1β (10ng/ml) caused an average 21% increase in GAG release from control levels, whereas equine IL-1β caused a 27% increase. Thus, equine IL-1β induces greater GAG loss from equine explants compared to human IL-1β at an equivalent concentration (10ng/ml) in the model and is therefore a suitable stimulus for inducing GAG loss.

Curcumin at 50μM and above significantly reduced IL-1β-stimulated GAG loss. Previously, we observed that curcumin (100μM) significantly reduced human IL-1β-stimulated GAG release from equine cartilage explants, but this effect was not seen at 10μM. This effect may be initiated at 50μM but it must be remembered that the previous study used human IL-1β on equine explants, which induced lower levels of GAG release compared to the equine IL-1β used in the current study.

Inflammatory mediators play a critical role in the development and progression of OA. PGE_2 levels are significantly increased in osteoarthritic joints compared to normal joints (Kirker-Head *et al.*, 2000) and for this reason it was chosen as a marker of inflammation in this study. IL-1β significantly increased the release of PGE_2 from

normal equine articular cartilage explants compared to non-stimulated controls, consistent with previous equine explant studies (Takafuji *et al.*, 2002; Petrov *et al.*, 2005).

The anti-inflammatory effects of curcumin (Jagetia and Aggarwal, 2007) have generated increasing interest in its potential for the treatment of inflammatory diseases. Curcumin significantly reduced IL-1β-stimulated PGE_2 release at all concentrations tested, from 25μM and above. Curcumin is thought to exert its anti-inflammatory effects through reducing COX-1 (Handler *et al.*, 2007), COX-2 (Hong *et al.*, 2004) and mPGES expression (Moon *et al.*, 2005), thus preventing PGE_2 release. This is most likely due to its inhibitory effect on the upstream NF-κB-signalling pathway which promotes PGE_2 production via up-regulating the COX and PGES genes (Catley *et al.*, 2003). Other studies have found significant anti-inflammatory effects of curcumin on other cells types at lower concentrations. No curcumin concentrations tested in this study failed to reduce IL-1β-stimulated PGE_2 release, thus it is likely that concentrations lower than 25μM are likely to exert anti-inflammatory effects on cartilage explants in this model.

With regard to the PGE_2 assay, the method used was the normal sensitivity option, which detected PGE_2 levels in the culture media of between 39 – 2050pg/ml. However, all the samples contained less than 1200pg/ml, meaning that measurements were read from the lower part of the curve. Therefore, future studies should use the high sensitivity method, which has a range of 19.6-1250pg/ml.

Interestingly, IGF-1 was not effective in the model, in that it did not significantly reduce IL-1β-stimulated GAG release, as had been observed previously. This indicated that the positive control had failed in the model. However, it could be seen that the data were consistent in that the explants responded to IL-1β and that

curcumin reduced both GAG and PGE_2 release from the IL-1β-stimulated explants. This suggested that the model was still working and so it was IGF-1 that had failed. Suggested reasons for the inability of IGF-1 to reduce IL-1β-stimulated GAG release from the explants included reduced performance due to long term freezer storage and the change from human to equine IL-1β which may have reduced the efficacy of the human IGF-1. In addition, there is thought to be an age related decline in the responsiveness of chondrocytes to IGF-1 which is mediated in part by an increase in IGF binding proteins (Martin *et al.*, 1997). Therefore, as the ages of the animals used in the study were unknown, it is possible that age may account for the lack of effect of IGF-1. However, despite this, IGF-1 was not a suitable positive control for the new parameter of PGE_2. Thus, a new positive control capable of reducing both PGE_2 and GAG release was needed for the model.

2.7. Study 5. Vehicle Controls

Curcumin in DMSO effectively reduced IL-1β-stimulated GAG and PGE_2 release from the cartilage explants. However, DMSO has been found to have a detrimental effect on cartilage explant integrity, causing reduced proteoglycan synthesis after 3 days in both bovine cartilage at 10% (Matthews *et al.*, 1998), and in equine cartilage at 5% and above (Smith *et al.*, 2000). Thus, it was important to carry out DMSO controls in order to determine whether the anti-catabolic and anti-inflammatory effects of curcumin were accurate or due to the DMSO vehicle. Vehicle controls containing the maximum volume of DMSO present in the wells of the highest concentration of curcumin (100μM) were used to elucidate whether observed effects with curcumin treatment were due to the solvent.

2.7.1. Materials and Methods

Study Design

Cartilage explants were collected from the MCP joints from a six-year-old horse as and acclimated in the incubator overnight. Culture medium was prepared as before and used as a control and to dilute all IL-1β, DMSO and curcumin stocks. Two batches of curcumin (100μM) were prepared from a 1mM stock (diluted down in culture medium from a frozen 100mM stock); one batch was made up in the culture medium alone, and the other in culture medium containing recombinant equine IL-1β (10ng/ml). Similarly, equivalent volumes of DMSO (to those in the curcumin 100μM treatments) were prepared in both plain and IL-1β-treated culture media, where the total volume of DMSO was 0.01% of the total 1ml volume in the well. A plain control and IL-1β alone treatment was also included to verify that the batch of IL-1β used was effectively inducing GAG release in the model. A GAG assay was performed as previously described (page 53).

Statistical Analysis

Percentage GAG release data from each treatment in triplicate were combined and a one-way between-groups ANOVA was conducted to test for a statistically significant difference at the $p<0.05$ level. *Post hoc* comparisons using the Tukey test were conducted to determine where the differences among the groups occurred. Values are reported as means of three replicates per treatment from one horse ± SEM.

2.7.2. Results

Control explants released a mean GAG percentage of 15.67 ± 0.39% into the culture media over five days (figure 19).

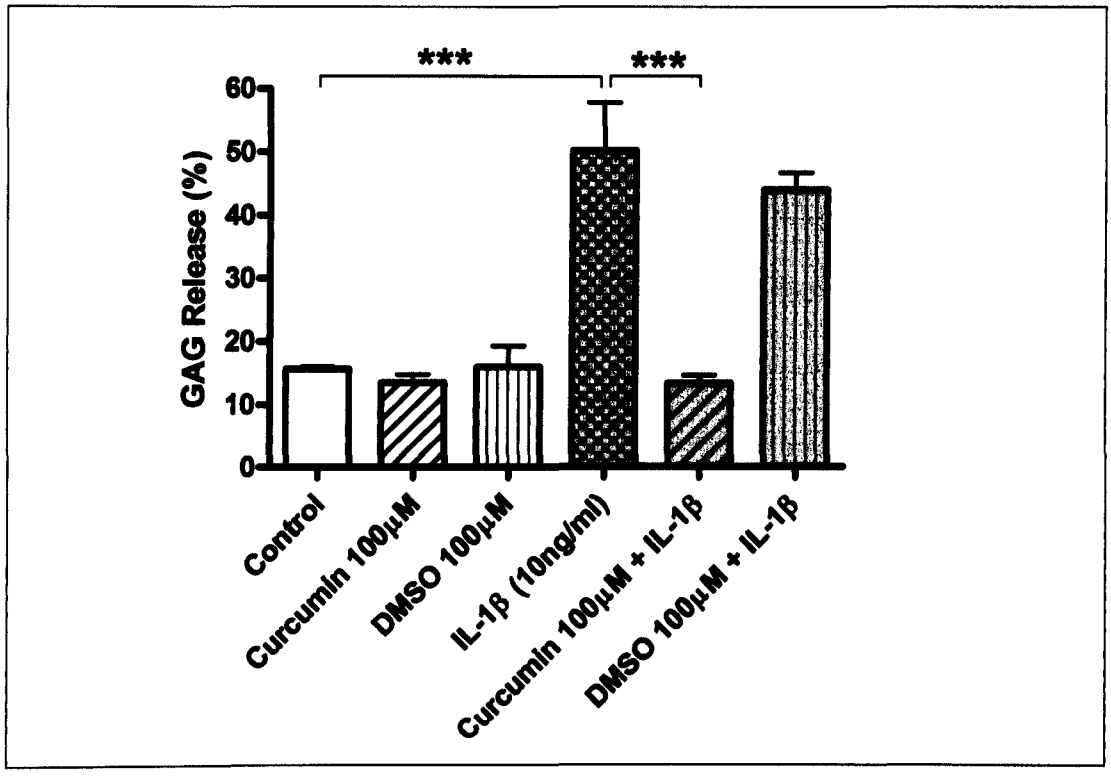

Figure 19. Percentage release of glycosaminoglycans (GAGs) in response to curcumin (100µM) or an equivalent concentration of dimethyl sulfoxide (DMSO) either with recombinant equine interleukin-1beta (IL-1β; 10ng/ml; grey columns) or without (clear columns)

*Significance compared to IL-1β is indicated by *** ($p<0.001$). Graphs give mean values collated from replicate wells (3 replicates per treatment) from one horses and bars represent standard error of the means (SEM). Control indicates cartilage discs incubated in the culture medium alone.*

Neither curcumin (100μM) nor the equivalent volume of DMSO (100μM) alone significantly affected this basal level of GAG release (13.50 ± 1.35% and 15.97 ± 3.38% respectively). IL-1β (10ng/ml) significantly increased GAG release to 50.27 ± 7.56% ($p<0.001$). This IL-1β-stimulated GAG release was significantly reduced by curcumin (100μM) to 13.33 ± 1.24% ($p<0.001$), whereas the equivalent concentration of DMSO (100μM) had no significant effect (44.00 ± 2.70, n.s).

2.7.3. Discussion

DMSO is a polar solvent that is commonly used to solubilise hydrophobic drugs and phytochemicals, including curcumin (Elattar and Virji, 2000; Balakin *et al.*, 2006). DMSO can aid the permeability of membranes and the transport of substances through membrane barriers without damaging them and so is used as a vehicle for both topical and oral medications (Jacob *et al.*, 1964; Jacob and Herschler, 1986). However, DMSO itself has a number of pharmacologically relevant properties including anti-inflammatory and anti-oxidant activity (Santos *et al.*, 2003), and reduced proteoglycan synthesis in cartilage explants (Matthews *et al.*, 1998). Thus it was necessary to determine the effects of DMSO in the model, to ensure that it was not responsible for the effects observed with curcumin.

This study showed that at the highest concentration of curcumin tested (100μM), the equivalent volume of DMSO (amounting to 0.01% DMSO) did not produce a significant decrease in IL-1β-stimulated GAG release. This suggested that DMSO was not interfering with the effects seen with curcumin treatment.

Although a previous study found DMSO (5%) reduced proteoglycan synthesis in cartilage explants, the same study also found that culturing explants with 1% DMSO

for up to 72 hours did not significantly alter proteoglycan synthesis compared to controls (Smith *et al.*, 2000). Therefore, although proteoglycan synthesis was not measured in this study, the low levels of DMSO used (0.01%) suggest that it was unlikely to be affecting proteoglycan synthesis. Thus, it was concluded that the reduction of IL-1β-stimulated GAG release observed in the curcumin-treated explants in the model was not due to the presence of DMSO. Subsequent studies did not include DMSO controls due to space restrictions on the culture plates. However, they used same batches of DMSO and curcumin, as used in this study, for continuity.

2.8. Study 6. Final Test of the Equine IL-1β Model Using Curcumin and Carprofen

The failure of IGF-1 to reduce IL-1β-stimulated GAG release in study 4 lead to the investigation of an alternative positive control. Due to space restrictions on the plate, it was necessary to find a single positive control that could reduce both IL-1β-stimulated GAG and PGE_2 release. In addition, the positive control needed to be a conventional pharmacological treatment that is a realistic and widely used treatment for patients, rather than IGF-1, which is not a practical treatment option. After consulting with a veterinarian and studying the literature, the NSAID, carprofen was selected as a positive control, due to its anti-inflammatory activity and beneficial effects on cartilage metabolism (Benton *et al.*, 1997; Armstrong and Lees, 1999). Control wells were distributed around the plate to monitor within plate variability.

The aim of the study was to investigate further the anti-inflammatory and anti-degradative effects of curcumin at concentrations between 3μM and 50μM using carprofen as the positive control. The proteolytic enzyme, MMP-3, is a catabolic

mediator of cartilage degradation and is present in its active form at higher levels in the synovial fluid from osteoarthritic joints compared to normal joints (Brama *et al.*, 2000c). Curcumin has been previously shown to reduce IL-1β-stimulated MMP-3 levels in human chondrocytes cultured in monolayers (Schulze-Tanzil *et al.*, 2004). Therefore, the levels of MMP-3 released into the culture media of the cartilage explants were investigated alongside GAG and PGE_2 release.

2.8.1. Materials and Methods

Study Design

Three horses (two pony types and one cob type) were used for the study. Both the MCP and MTP joints were used due to the joints being small and limiting the cartilage harvested from them. Explants were incubated in DMEM containing recombinant equine IL-1β (10ng/ml) and various concentrations of curcumin (3μM, 6μM, 12μM, 25μM and 50μM) prepared in IL-1β-treated media as previously described (page 65). The NSAID carprofen, Rimadyl® (Pfizer), (100μg/ml) was prepared in IL-1β-treated media and included as a positive control.

All plates contained 1ml culture media, which formed the base for other treatments and acted as a control for each plate. Plates were incubated at 37°C and 5% CO_2 for 5 days. After 5 days, explants and the corresponding media were frozen at -20°C. Samples underwent a GAG assay, carried out as previously described (page 53), as well as a PGE_2 assay and analysis of MMP-3 by western blotting.

PGE_2 Assay (High Sensitivity)

A competitive immunoassay kit, as used previously, was used to measure PGE_2 according to the manufacturer's instructions. Samples from previous studies were found to contain under 1200pg/ml PGE_2, therefore the high sensitivity procedure was undertaken to improve accuracy. In brief, standards (19.6 - 1250pg/ml) and supernatant samples (150µl) were added to a 96-well plate coated in goat anti-mouse polyclonal antibody, followed by primary antibody solution (50µl) and PGE_2 conjugate (50µl), then incubated for 19 hours at 6°C. The plate was thoroughly washed before adding 200µl substrate solution to each well and developing in the dark at room temperature for 20 minutes. A stop solution (50µl) was added and the plate was read immediately on a plate reader at 450nm with wavelength correction set at 540nm using Ascent Software (version 2.6).

Protein Quantification of Samples

The protein content of explant supernatants from the GAG and PGE_2 studies was quantified using a detergent compatible (DC) protein assay (Bio-Rad Laboratories), similar to the Lowry assay (Lowry *et al.*, 1951). It is based on the reaction between protein and copper in an alkaline solution and the subsequent reduction of Folin reagent by the copper-treated protein.

Briefly, protein standards (0.2, 0.5, 0.75, 1, 1.5mg/ml) were prepared in DMEM. Standards and samples (5µl) were added to the appropriate wells of a microtiter plate. An alkaline copper tartrate solution (25µl) was then added to each well, followed by Folin Reagent (200µl). The plate was shaken for 5 seconds then left for 15 minutes. Absorbance was read at 720nm.

The protein content of each sample was calculated and divided into 50μg aliquots. The aliquots were freeze-dried (Heraeus-Christ) overnight before preparing for western blotting the following day.

Western Blotting of MMP-3

The resultant pellets were resuspended in 37μl sample buffer (NuPAGE lithium dodecyl sulphate (LDS) sample buffer (1X)) and electrophoresed on precast 4-12% Bis-Tris 10-well gels (Invitrogen) under denaturing and reducing conditions. Proteins were transferred to a 0.45μm Polyvinylidene fluoride (PVDF) membrane and blocked with 5% (w/v) non-fat milk with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween20 for one hour. The membranes were incubated with a goat polyclonal antibody raised against the full length human MMP-3 protein (Abcam) diluted 1:1,000 in 5% (w/v) non-fat milk at 4°C overnight. Membranes were then washed and incubated for 2 hours at room temperature with a secondary anti-goat antibody (1:10,000; Dako). Membranes underwent a final 30 minute wash before chemiluminescence was detected using ECL+ (GE Healthcare) on a Typhoon Trio+ Variable Mode Imager (GE Healthcare). Densitometric quantification of the MMP-3 bands was performed using ImageJ software (National Institutes of Health). The relative intensity of the bands in comparison to controls was measured for samples from each animal.

Statistical Analysis

Data from all horses in the experiment were combined (either percentage GAG release, PGE_2 values in pg/ml, or relative intensity of western blotting bands

compared to control) and a one-way between-groups ANOVA was conducted to test for a statistically significant difference at the $p<0.05$ level. *Post hoc* comparisons using the Tukey test were conducted to determine where the differences among the groups occurred.

2.8.2. Results

Curcumin (3-50μM) Significantly Decreases IL-1β-Stimulated GAG Release

Control explants released 11.38 ± 1.26% of the total GAG content of the cartilage into the media over five days (range: 86-323μg GAG/ml of culture supernatant) (figure 20).

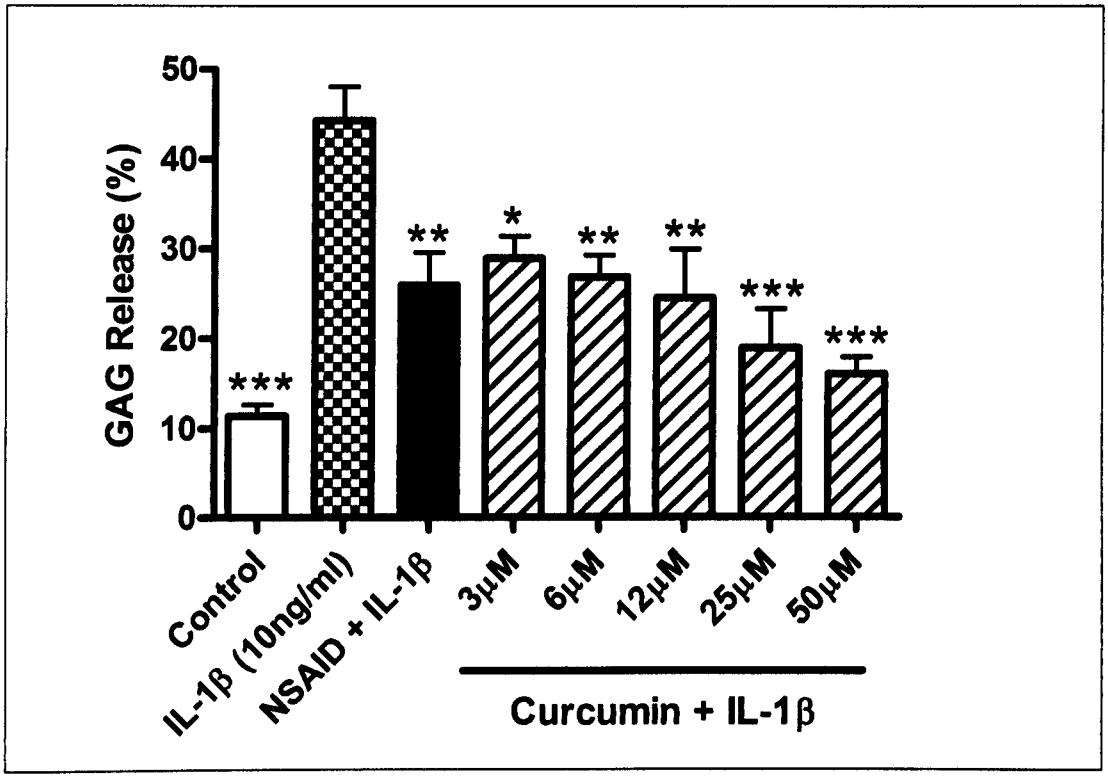

Figure 20. Percentage glycosaminoglycan (GAG) release from recombinant equine interleukin-1beta (IL-1β)-stimulated cartilage explants treated with a nonsteroidal anti-inflammatory drug (NSAID; 100μg/ml) or curcumin (3-50μM)

*Control column indicates cartilage discs incubated in the culture medium alone. Values are reported as the mean of 3 replicates of 3 horses per treatment and bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml) is indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).*

IL-1β (10ng/ml) significantly increased GAG release to 44.31 ± 3.75% of total GAG content compared to control ($p<0.001$). The NSAID at 100μg/ml significantly reduced IL-1β-stimulated GAG release to 25.96 ± 3.59% of total GAG content ($p<0.01$). Curcumin significantly decreased equine IL-1β-stimulated GAG release in the explants to 28.98 ± 2.45% at 3μM ($p<0.05$), 26.84 ± 2.49% at 6μM ($p<0.01$), 24.51 ± 5.42% at 12μM ($p<0.01$), 18.91 ± 4.36% at 25μM ($p<0.001$) and 16.05 ± 1.82% at 50μM ($p<0.001$).

Curcumin (3-50μM) Significantly Reduces IL-1β-Stimulated PGE_2 Release

PGE_2 release into the media of unstimulated explants was 20.35 ± 3.67pg/ml (figure 21).

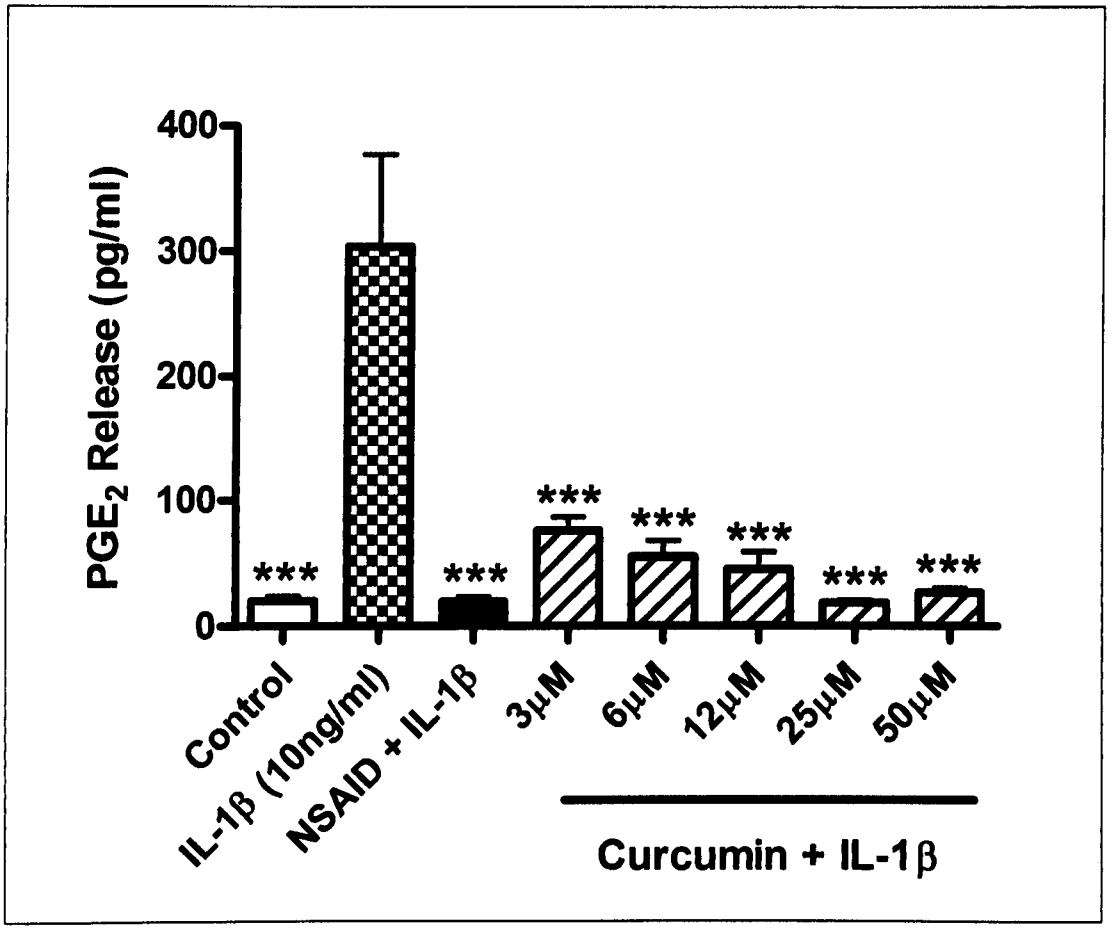

Figure 21. Prostaglandin E_2 (PGE_2) released from recombinant equine interleukin-1beta (IL-1β)-stimulated cartilage explants treated with a nonsteroidal anti-inflammatory drug (NSAID; 100μg/ml) or curcumin (3-50μM)

*Control column indicates cartilage discs incubated in the culture medium alone. Values are reported as the mean of 3 replicates of 3 horses per treatment and bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml) is indicated by *** ($p<0.001$).*

These levels were significantly increased ($p<0.001$) to 303.9 ± 73.38pg/ml by the addition of recombinant equine IL-1β (10ng/ml). The NSAID significantly attenuated this effect, reducing levels to 19.72 ± 3.74pg/ml ($p<0.001$). Curcumin also significantly reduced IL-1β-stimulated PGE_2 release to 75.48 ± 10.68pg/ml at 3μM ($p<0.001$), 54.72 ± 12.41pg/ml at 6μM ($p<0.001$), 45.65 ± 13.31pg/ml at 12μM ($p<0.001$), 18.36 ± 2.38pg/ml at 25μM ($p<0.001$) and 26.73 ± 3.52pg/ml at 50μM ($p<0.001$).

Curcumin (12-50μM) Reduces IL-1β-Stimulated MMP-3 Release

Control explants released low levels of MMP-3, which were significantly increased by the addition of IL-1β ($p<0.001$) (figure 22).

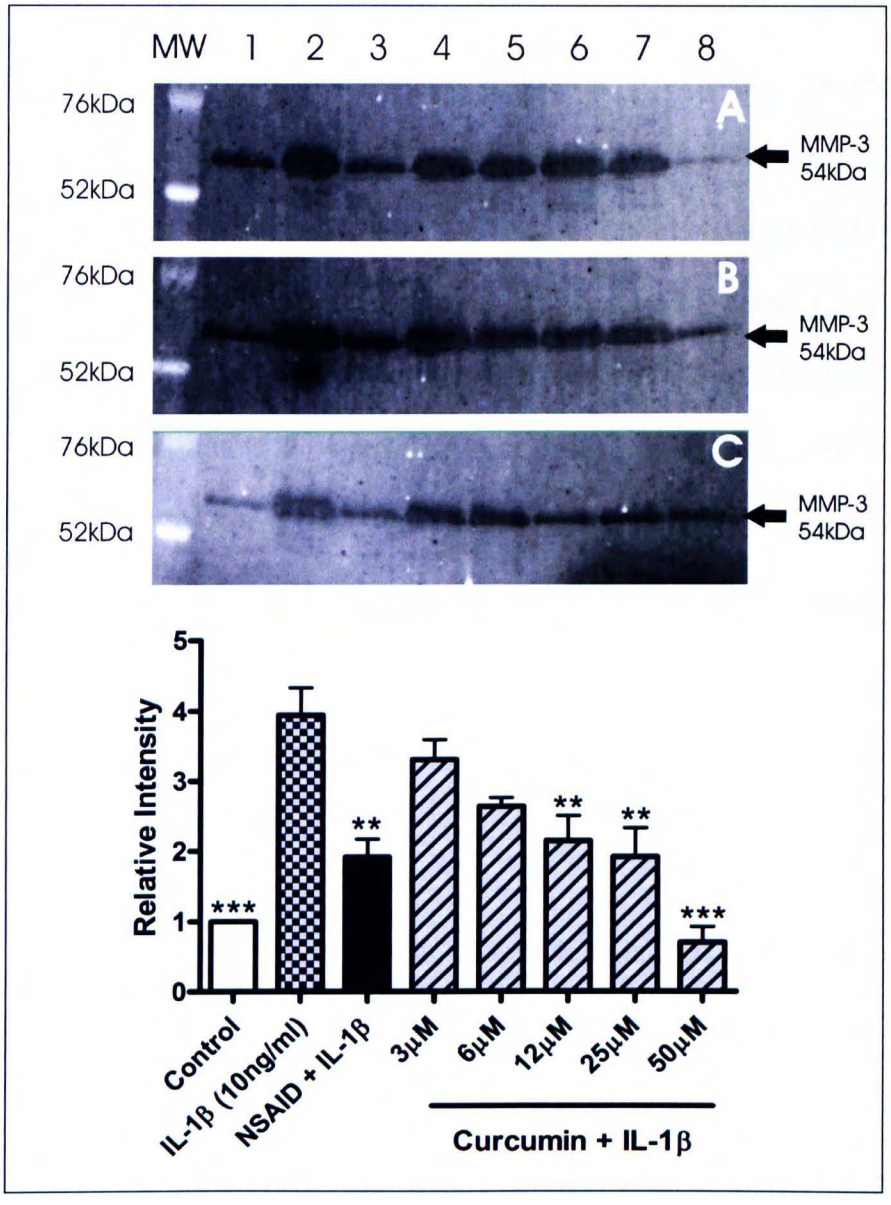

Figure 22. Western blot quantification of matrix metalloproteinase (MMP)-3 release from cartilage explants cultured with recombinant equine interleukin-1beta (IL-1β; 10ng/ml) and a nonsteroidal anti-inflammatory drug (NSAID; 100μg/ml) or curcumin (3-50μM)

*Marker lane shows the molecular weights (MW) in kilodaltons (kDa). Graph shows the relative intensity of bands from 3 horses (A, B, C. Values are reported as the mean of 3 horses per treatment and bars represent standard error of the means (SEM). Significance compared to interleukin (IL)-1β is indicated by ** ($p<0.01$) and *** ($p<0.001$). Lane 1 is the control. The remaining lanes contain IL-1β (10ng/ml) either alone (lane 2), or with a NSAID (100μg/ml; lane 3), or with curcumin at 3μM (lane 4), 6μM (lane 5), 12μM (lane 6), 25μM (lane 7) and 50μM (lane 8).*

The addition of the NSAID significantly reduced IL-1β-stimulated MMP-3 levels ($p<0.01$) in all animals to near that of controls. Curcumin showed a dose-dependent significant effect on reducing the IL-1β-stimulated release at 12μM ($p<0.01$), 25μM ($p<0.01$) and 50μM ($p<0.001$). However, the concentration at which this reduction became apparent differed between animals. For example, horse C, showed a reduction in pro-MMP-3 secretion at curcumin concentrations of 12μM and above, whereas in horses A and B, the equivalent reduction was not seen until curcumin concentrations of 50μM were used. The most apparent reduction in pro-MMP-3 secretion was always seen at 50μM with levels near to that of the controls in all animals.

2.8.3. Discussion

This study set out to extend the knowledge of the biological actions of curcumin by determining whether it could inhibit IL-1β stimulated cartilage degradation and inflammatory mediator production.

IL-1β (10ng/ml) significantly increased GAG, PGE_2 and MMP-3 release in all horses as expected. Gene expression of MMP-3 is significantly down-regulated in late-stage OA cartilage samples compared to early stage-OA samples (Bau *et al.*, 2002). Therefore, the increased level of MMP-3 protein in the IL-1β-treated samples from this study supports the genomic data and the idea that the model represents early OA.

The NSAID significantly reduced GAG, PGE_2 and MMP-3 release in all horses suggesting that it is a suitable positive control for these quantitative methods. This is in agreement with a study reporting the anti-inflammatory and anti-catabolic effects of carprofen in equine cartilage explants stimulated with human IL-1β (Armstrong

and Lees, 1999). Although there has been conflicting evidence with regard to the anti-catabolic effects of carprofen with one study finding that it did not reduce GAG release from canine cartilage explants, the cartilage was taken from dogs undergoing hip-replacement surgery, not from clinically normal animals as used in the present study (Benton *et al.*, 1997). This reflects the value of carprofen in early OA, which is supported by evidence that carprofen reduced the progression of early structural changes (in terms of osteophyte size, and lesion size and severity) in a canine model of OA (Pelletier *et al.*, 2000). In light of the present results, it was decided that carprofen (Rimadyl®) was an appropriate positive control for all the tested parameters in the model.

Curcumin at 3μM and above significantly reduced IL-1β stimulated GAG loss from cartilage explants. Previous studies in this chapter showed that curcumin (50μM) significantly reduced human IL-1β-stimulated GAG release from equine cartilage explants, but this effect was not evident at 25μM. The concentration differences between these two studies may be linked to individual variation as curcumin (25μM) reduced IL-1β-stimulated GAG loss to a mean of 18.91% in the present study, compared to 32.45% in the previous study. The high mean GAG release for the three horses in the previous study was due to explants from one horse releasing 57.02% mean GAG, whereas the other two horses had lower mean GAG levels (20.96% and 16.98%), similar to the mean value of the combined horses in the present study. This high value was included in the analysis, as the horse responded to IL-1β with a significant increase in GAG release, and showed a dose-dependent reduction in IL-1β-stimuated GAG loss with increasing curcumin concentrations (although levels were still higher than those of the other two horses). This suggests that individual

variation in response to curcumin may be responsible for the concentration differences between studies.

The involvement of pro-inflammatory cytokines and MMPs in OA is well documented (Shinmei *et al.*, 1991; Chubinskaya *et al.*, 1996). MMP-3 is a stromelysin produced by chondrocytes in OA cartilage tissue (Okada *et al.*, 1992). MMP-3 gene expression is up-regulated in response to IL-1β stimulation in chondrocytes (Tung *et al.*, 2002). Similarly, MMP-3 protein expression is increased by the addition of IL-1β to cartilage explants (Julovi *et al.*, 2004). MMP-3 was chosen as a marker of cartilage explant degradation in this study as there is convincing evidence for a role for MMP-3 in cartilage destruction in OA. Curcumin reduced MMP-3 secretion at concentrations as low as 12μM in some animals, and as high as 50μM in others, suggesting some animal-to-animal variability. However, when treated with 50μM curcumin, IL-1β-stimulated explants from all animals secreted MMP-3 levels lower than, or equivalent to, unstimulated control explants. This is in agreement with a previous study showing that curcumin (50μM) effectively reduced MMP-3 levels in IL-1β-stimulated-human chondrocyte lysates (Schulze-Tanzil *et al.*, 2004). However, lower curcumin concentrations have been reported as effective in cartilage from human OA patients post mortem, where curcumin reduced MMP-3 activity in the media of IL-1β-stimulated chondrocytes at 15μM and in cartilage explants at 5μM (Mathy-Hartert *et al.*, 2009). Many variables may account for the difference in effective curcumin concentrations, including individual variation, pre-existing joint pathology, the explant model used, and the fact the latter study looked at MMP-3 activity rather than the presence of the protein by western blotting. It should be noted that many MMPs, including collagenases, matrilysin (MMP-7), and other stromelysins (e.g. MMP-10), are involved in

osteoarthritic cartilage degradation (Mitchell *et al.*, 1996; Ohta *et al.*, 1998; Barksby *et al.*, 2006). Thus, the reduction in MMP-3 secretion would contribute to, but not totally account for, the reduction in GAG release from IL-1β-stimulated cartilage explants. However, further work to determine the levels of activated MMP-3 in the samples is needed to confirm this.

The reported anti-inflammatory effects of curcumin (Jagetia and Aggarwal, 2007) have stimulated increasing interest in its potential for the treatment of inflammatory disorders. In this study, curcumin at concentrations of 3μM and over significantly reduced PGE_2 release in response to equine IL-1β (10ng/ml). This anti-inflammatory effect is consistent with previous work in other cell culture models, such as rat peritoneal macrophages where curcumin (10μM) inhibited PGE_2 release by 45% (Joe and Lokesh, 1997), and in BV2 microglial cells where curcumin (10μM and 20μM) significantly reduced PGE_2 release in response to LPS (0.5μg/ml) (Jin *et al.*, 2007). The reduction in PGE_2 levels in response to IL-1β in this chapter is postulated to be due to the inhibitory effects of curcumin on the NF-κB pathway. Curcumin (50μM) has been shown to inhibit various steps of the NF-κB pathway, such as IL-1β-dependent phosphorylation of p65; nuclear-translocation of p65; and IκBα phosphorylation in IL-1β-stimulated human chondrocytes (Shakibaei *et al.*, 2007). Thus, by inhibiting NF-κB signalling, curcumin prevents the downstream inflammatory effects of COX-2 expression and synthesis. This partly explains the anti-inflammatory effect of curcumin in response to LPS which activates the NF-κB pathway by activating toll-like receptors (TLRs) (Faure *et al.*, 2000; Kawai and Akira, 2007). In support of this, curcumin (4μM-16μM) has been shown to significantly reduce LPS-induced COX-2 expression in BV2 microglial cells by inhibiting DNA binding of NF-κB and AP-1 (Kang *et al.*, 2004). However,

curcumin also reduces LPS-induced inflammation by preventing the dimerization of TLR4 required for activating downstream signalling pathways (Youn *et al.*, 2006). Thus, the inhibitory effects of curcumin on different pathways at multiple levels, highlights its potential for supporting joint health in response to a variety of inflammatory stimuli.

In conclusion, this study added supporting evidence as to the suitability of using equine IL-1β (10ng/ml) in the model due to its significant effects on increasing GAG, PGE_2 and MMP-3 release from explants. The results of the NSAID, carprofen, indicate that it is a suitable positive control, although further studies are needed with more horses to confirm its consistency in repeatedly reducing IL-1β-stimulated GAG release.

2.9. Chapter Discussion

2.9.1. The Explant Model

The cartilage explant system is a well-established *in vitro* model, which facilitates the study of chondrocytes in their native matrix. It is a very versatile model, enabling comparisons between cartilage from normal and OA patients (Lafeber *et al.*, 1993), as well as using various stimuli to create OA-like structural changes in healthy cartilage (Patwari *et al.*, 2003). It is applicable to multiple species (e.g. canine, porcine, bovine, equine, human) either to study in their own right or to be used as a model for other species, where material from the species of interest is lacking or difficult to obtain. The model can also utilise different cartilage types from different anatomical locations (e.g. nasal cartilage, or articular cartilage from various articulating joints) although these two types of hyaline cartilage differ in

matrix composition and response to IL-1α (Billinghurst *et al.*, 2000; Jansen *et al.*, 2010). For the purpose of this thesis, equine cartilage was chosen for two reasons, firstly because the focus of the thesis was equine. Secondly, the horse is a suitable model of human OA, owing to the fact that horses are athletic animals that can suffer from naturally occurring injuries similar to those of human athletes (Koch and Betts, 2007). There are also significant similarities in joint cartilage composition between the two species (Frisbie *et al.*, 2006). However, the disadvantage of using equine cartilage is the natural variation that is inherent with a non-production animal. Horses are utilised for a diverse range of sport and leisure purposes with varied exercise intensities. Equine euthanasia may be selected due to injury (resulting in loss of performance or permanent discomfort) or for financial reasons. Thus, horses entering British abattoirs vary widely in age, breed, history of use and reason for euthanasia. In contrast, meat-production animals are often of similar breed, experience similar animal husbandry practices and are slaughtered before they reach a certain age. Although the variability in equine material could have been reduced by breeding horses specifically for the study, this was not an option due to ethical and financial constraints. Utilising abattoir material and horses brought to the veterinary school for non-research related euthanasia, meant that no horses were sacrificed for the work in this thesis. This was an important ethical requirement of the commercial sponsor. Therefore, a large variety of horses were used in the studies in this thesis and the variability of the cartilage obtained from them resulted in the setting of inclusion criteria for the model as it developed.

2.9.2. Subject Age

Age has a marked effect on cartilage metabolism, with cartilage from older horses being less responsive, in terms of GAG synthesis and release, to IL-1α stimulation (MacDonald *et al.*, 1992; Morris and Treadwell, 1994). These studies do differ in their classification of 'aged' animals and in anatomical locations of cartilage collection, i.e. the carpal joints of one 14-year-old stallion (Morris and Treadwell, 1994) and the MTP joints of two 20-year-old geldings (MacDonald *et al.*, 1992). Although these studies used IL-1α instead of IL-1β and used different joints, this age-associated decreased responsiveness to cytokines highlights the importance of considering age when using the explant model. Many of the studies described in this chapter did not account for age. After the completion of this chapter, a different source of equine material was discovered, where at the time of euthanasia, the age of the horse was determined from examining the teeth. Thus, inclusion criteria for the model was set on Thoroughbred-type animals under the age of 12-years-old as they were the most common breed-type in the abattoir and were of a similar weight range.

2.9.3. Animal Numbers

In the model, each animal was analysed individually and then animals were combined to assess whether the overall results reflected the trends seen in individual animals. Although some of the earlier studies used more than three horses, the time-consuming nature of processing and collecting samples meant that, in the interests of consistency, three horses were used per study. This meant that nine values were obtained for each treatment group (three replicates from three horses). This number of animals is more than some used in other studies looking at the effects of a

treatment on the GAG content of cartilage, for example using only one horse with four replicates per treatment (Takafuji *et al.*, 2002) or two horses with 4 replicates (Dechant *et al.*, 2005). Some studies have used 10 horses, with three replicates per treatment (Petrov *et al.*, 2005). However, initial studies with larger numbers of horses found that three horses were enough to indicate a trend and achieve significance. Therefore, in the interests of consistency and accuracy, three horses were used per study.

2.9.4. Joint Selection

Previous studies have highlighted the variation between joints, for example, cartilage catabolism, in terms of GAG release, in the MCP joints is relatively lower than in the proximal interphalangeal and distal interphalangeal joints (Fuller *et al.*, 2001). Thus, studying a similar anatomical region is of importance in the model. Consequently, the MCP and MTP joints were selected for the model, due to their weight bearing properties and the fact that they have a wide range of motion and are a common site for equine OA. To reduce variation further, it was decided that future studies would only use the MCP joints in the explant model, unless the cartilage yield was exceptionally low.

Another criteria for inclusion included macroscopically normal joints as determined by observation of a smooth intact cartilage surface, clear viscous synovial fluid and a non-inflamed synovium. This was supported with use of histology. Twenty-eight histological samples were collected from the MCP, MTP, carpal and tarsal articular surfaces of the joints from ten horses whose joints appeared suitable (at the macroscopic level) for use in the model (appendix 4). Samples were declared

microscopically suitable for the model if they were free of lesions in the articular surface. This established that the correct samples were being identified, so subsequent samples were not routinely processed for histology, but were collected and stored for future reference.

2.9.5. Cartilage Pooling and Explant Selection

Cartilage from an individual animal was pooled for that animal. However, cartilage from different horses was kept separate, to enable the individual responses of each animal to be assessed. Pooling animals would have affected the results by increasing variation and masking the individual effects.

Explants were excised from cartilage slices with a 3mm biopsy punch and explants were distributed around the culture wells at random (three explants per well) to ensure that there was a variety of explants from different cartilage slices in each well. Explant studies in the literature have used differing sizes of explants ranging from 1mm cubes (Fuller *et al.*, 2001) to 8mm discs (Parkkinen *et al.*, 1992). However, the size of the donor species and the target joint often restrict the size of the explants that can be obtained. Several different sized explants were collected during the thesis and it was found that 3mm discs allowed the desired number of explants per well to be obtained without wasting cartilage or compromising uniformity in thickness as found in larger explants. Explant diameters of 3mm have been used previously for evaluating GAG release (Patwari *et al.*, 2003). Thus 3mm discs were selected for the model.

2.9.6. Culture Period

For the model, harvested cartilage explants were placed in culture medium without FBS overnight to acclimate and increase the stability of cultures. Damage to the cartilage matrix, either in OA or by the process of cutting, decreases the tension of the collagen network, consequently allowing the tissue to swell and allowing peripheral GAGs to leach out (Maroudas, 1976; Urban and Maroudas, 1981). Thus, overnight acclimation media were removed from the wells to reduce any residual effects from the explant harvesting procedure. Following this acclimation period, the media were replaced with media containing the treatments for five days. A five day culture period was selected because it allowed enough time to see beneficial effects in the model without having to change the media and alter the steady state environment of the cultures. Although the lack of time course data could be criticised in the studies, this has been addressed previously (Bird *et al.*, 1997; Williams *et al.*, 2003), and the objective of this study was not to monitor the kinetics of GAG release, but to compare GAG release at five days in comparison to control cartilage.

2.9.7. Serum-Free Culture

With regard to culturing conditions, there is conflicting evidence over the use of foetal bovine serum (FBS) in explant models. The presence of serum in culture medium can cause chondrocytes to change their phenotype (Malpeli *et al.*, 2004) and cartilage explants to lose their biochemical and mechanical properties (Bian *et al.*, 2008). Thus it appears culturing without serum would be preferable, however some studies report that proteoglycan synthesis is reduced without serum and there is a

gradual loss of proteoglycans from the tissue (Hascall *et al.*, 1983; MacDonald *et al.*, 1992; Kawcak *et al.*, 1996). This is in contrast to other studies showing that cartilage explants did not decrease their GAG content after a six week period in serum-free culture (Bian *et al.*, 2008). Culturing cartilage explants in serum-free medium has distinct advantages over culturing with FBS as it removes the confounding factors of adding endogenous cytokines and proteases present in FBS and the natural batch variability of its constituents. Thus, in the interests of consistency, it was decided that serum-free culture conditions should be used in this model. Despite the steady proteoglycan loss that has been reported with serum-free cultured explants, expressing GAG release as a percentage of the total GAG available allows this loss to be monitored in control explants cultured in medium alone, before comparing to treated explants. Taken as a whole, all the explants used as controls in all the studies in this chapter (66 values) released an average of 11.46 ± 0.51% (GAG ± SEM) over five days (appendix 5). More often than not, this was under 10% but a few higher values increased the mean. Previous work with equine MCP/ MTP cartilage explants cultured in serum-free media has shown that control explants (incubated in serum-free media) release roughly 7% GAG over three days (Petrov *et al.*, 2005), and four days (Fuller *et al.*, 2001). Considering that the studies in this chapter quantified GAG release over five days and used different size of explant to those in the published studies, as well as taking into account the biological variation between horses, it can be seen that total GAG release is not markedly different from the values in the literature.

2.9.8. Measuring GAG Release in the Model

It should be noted when looking at the GAG loss data that these are only a measurement of net GAG loss. Cytokines such as IL-1β and TNF-α are known to reduce the synthesis of matrix proteoglycans, thereby limiting the reparative ability of cartilage (Dingle, 1984; Saklatvala, 1986; Taskiran *et al.*, 1994). No measurement of GAG synthesis was taken in these studies, which would show whether the material being tested could remove the inhibitory effect of IL-1β on GAG synthesis. Measuring both GAG synthesis and release would result in four different scenarios, which would determine whether the overall effect on the cartilage was positive or negative. For example, a product that increases GAG release is detrimental when coupled with reduced GAG synthesis, but in the presence of increased synthesis, it may simply represent increased matrix turnover. Likewise, a product that decreases GAG release would be beneficial in the presence of increased synthesis. However, if both GAG synthesis and release were reduced then this may represent a negative overall effect on the cartilage resulting from reduced metabolism. GAG synthesis in cartilage explants can be evaluated by measuring the incorporation of sulphur 35 (^{35}S)-labelled sodium sulphate into proteoglycans (Yaron *et al.*, 1989; Benton *et al.*, 1997; Bird *et al.*, 1997). Measuring GAG synthesis would be a beneficial future step to this work to gain a better understanding of GAG turnover in response to different plant extracts. However, time and radiation restrictions meant that the measure of GAG loss alone was chosen as an indicator of a beneficial effect for this thesis as has been used previously (Petrov *et al.*, 2005).

2.9.9. Units of GAG and PGE_2 Measurement

It can be argued that GAG results should be expressed as GAG (μg) per milligram of cartilage to account for variation in explant weight, as previously shown as either wet weight (Pratta *et al.*, 2003) or dry weight (Bird *et al.*, 1997). However, percentage GAG release has been used in previous studies (D'Lima *et al.*, 2001; Petrov *et al.*, 2005) and was selected as a more accurate measure. Expressing the results as a percentage meant that the proportion of GAG released in relation to the total content of GAG available per explant was quantified. Obviously, this is not the case when discussing the PGE_2 results but although the data are expressed as a total value of PGE_2 per ml, pilot studies showed that the explant weight variation was low, with a mean wet weight (± SEM) of 4.24 ± 0.10mg (appendix 6). Thus, the explants were of a similar weight and size. The assay used in these studies was able to detect a significant increase in PGE_2 in response to IL-1β and consequent reduction in response to an anti-inflammatory phytochemical after five days, effects which have been reported in equine explants stimulated with human IL-1β (10ng/ml) in response to an anti-inflammatory agent after three days (Petrov *et al.*, 2005).

2.9.10. Effects of Curcumin in the Model

With regard to using curcumin in the model, the level at which GAG release was reduced changed between studies. This is more than likely due to the changes that occurred in the model as it developed, such as the species-specificity of IL-1β and improved assay technique. However, the final study showed that low micromolar concentrations effectively antagonize GAG release *in vitro* and exerted a potent anti-inflammatory effect on cartilage explants treated with IL-1β. This shows that as the

model developed using curcumin, the assays were able to detect differences in GAG, PGE_2 and MMP-3 release consistent with the use of IL-1β. Many of the degradative and inflammatory effects of IL-1β are thought to be mediated by the NF-κB, mitogen-activated protein kinase (MAPK) and AP-1 signalling pathways, which increase the transcription of degradative MMPs and inflammatory COX enzymes resulting in PGE_2 production, amongst others (Liacini *et al.*, 2002; Kida *et al.*, 2005). Curcumin acts via inhibiting various stages of the NF-κB pathway including the inhibition of IκBα degradation and thus activation of the NF-κB complex and preventing the nuclear translocation of NF-kB and the up-regulation of degradative and inflammatory mediators (Schulze-Tanzil *et al.*, 2004; Shakibaei *et al.*, 2007). Hence curcumin was an appropriate phytochemical to test on IL-1β-stimulated cartilage. However, it must be considered that other test substances may block pathways that are not activated in this model. So although a material may not reduce IL-1β-stimulated PGE_2 or GAG release, it does not necessarily mean it would be ineffective as a joint health supplement. However, for the purpose of this thesis, product efficacy was based on its anti-inflammatory and anti-degradative effects in the model.

Although the aim of the study was to develop the model, the beneficial effects of curcumin on reducing GAG, MMP-3 and PGE_2 release at low micromolar concentrations were interesting and were a useful test of the model. The data generated from the model add support to the existing evidence suggesting that curcumin may be a suitable adjunct to conventional medicine for the treatment of inflammatory and degenerative disorders such as OA. However, 100μM is a very high concentration considering that previous studies have shown that curcumin (50μM) is toxic to a variety of cells such as astrocytes (Scapagnini *et al.*, 2002) and

synoviocytes (Jackson *et al.*, 2006). Therefore, there were concerns that the drastic reduction in GAG release seen at 100μM curcumin may have been a consequence of reduced metabolism in dead or dying chondrocytes. Consequently, cytotoxicity studies were needed to support the work carried out with curcumin in this chapter.

CHAPTER 3. ASSESSING CYTOTOXICITY OF CURCUMIN IN CHONDROCYTES

3.1. Introduction

Curcumin at concentrations between 50-100μM has been shown to have anti-inflammatory properties via its suppressive effects on IκB activity and consequently the NF-κB signalling pathway in various cell types, such as human intestinal epithelial cells and chondrocytes (Jobin *et al.*, 1999; Shakibaei *et al.*, 2007). Studies in the previous chapter confirmed the anti-inflammatory and anti-catabolic capabilities of lower concentrations of curcumin on IL-1β-stimulated cartilage explants. However, the significant effects of curcumin (100μM) on reducing IL-1β-stimulated GAG and PGE_2 levels to that of unstimulated controls could have been a consequence of cytotoxicity.

Curcumin is known to exert pro-apoptotic effects in cancer cells and cancer cell lines (Jiang *et al.*, 1996; Jee *et al.*, 1998; Chen *et al.*, 1999; Wang *et al.*, 2006). Consequently, it has been proposed as an anti-cancer chemotherapeutic agent. With regard to cartilage, during the course of this thesis, a paper was published indicating that curcumin was cytotoxic to transformed chondrocyte cell lines at 50μM (Toegel *et al.*, 2008). However, past research has indicated that curcumin exerts different effects on normal cells and 'transformed' or immortalised cancer cells (Syng-Ai *et al.*, 2004). Primary cells in their initial passages can be more phenotypically relevant than transformed cells (Finger *et al.*, 2003). Therefore, the cytotoxicity observed in chondrocyte cell lines may be a consequence of the transformation induced by the SV virus. Although virus-based transformation causes only a minor alteration at the genomic level, it has major consequences for the transcriptome and hence the

phenotype of the cells. However, it is clear that further research is required to determine the difference in curcumin cytotoxicity between healthy and diseased cells, as well as between primary cells and transformed cell lines. Thus, for this chapter, it was important to assess the viability of primary equine chondrocytes cultured with curcumin. Accordingly, concentrations at which a commercially available curcumin extract, previously shown to reduce cartilage loss and inflammatory mediator production in an *in vitro* model of early OA, were evaluated for toxicity towards primary equine chondrocytes. The studies in this chapter used a variety of methods to determine cytotoxicity using trypan blue staining, fluorescence-activated cell sorting (FACS) and a two-colour fluorescence cell viability assay. The aim of this was to produce a rapid and reliable method for detecting cytotoxicity of potential substances, in order to determine non-cytotoxic dose ranges to test in the explant model. The hypothesis to be tested was that non-toxic concentrations of curcumin could have beneficial anti-inflammatory effects in the explant model.

3.2. Materials and Methods

Subjects

Macroscopically normal articular cartilage samples were obtained from 11 horses (2-12 years old), of mixed breed and sex. Studies were carried out over five separate experiments due to horse tissue availability and the development of the method for detecting cytotoxicity. Thin cartilage shavings (less than 0.2cm in depth) were used for chondrocyte isolation from the animals in the first four studies. The final study used full depth cartilage for explant culture. Chondrocytes and cartilage explants

from each animal were kept separate throughout this study to account for inter-individual variability in response to curcumin. Cartilage shavings were aseptically harvested into low glucose DMEM containing 4% penicillin/streptomycin before washing in PBS containing 10% penicillin/streptomycin for 20 minutes.

Chondrocyte Isolation and Culture

Thin cartilage slices were digested overnight in 0.1% collagenase type I (Sigma-Aldrich) at 37°C and 5% CO_2. The resulting cell suspension was filtered and washed before undergoing first expansion in low glucose DMEM with 2% penicillin/streptomycin and 10% FBS. Once confluency was reached, cells were either passaged into 25cm^2 flasks or into 6- or 12-well plates. Only first, second and third passage confluent cells were used in this study.

Study Design – Monolayer Cultures for Morphology Pictures

Third passage chondrocytes from the MTP joints of a cob-type horse were grown to 95% confluence in 6-well plates. Culture medium containing serum was used as the control and as the base for the other treatments. Frozen stock solutions of curcumin (100mM; C1386, Sigma-Aldrich) that had been previously prepared in DMSO were defrosted and diluted in DMEM to 1mM. From this 1mM stock, test concentrations of curcumin (25μM, 50μM, 75μM and 100μM) were prepared in DMEM and added to the appropriate wells. Ice cold methanol (70%) was added as a positive control for cell death. The plates were incubated overnight at 37°C and 5% CO_2 for 24 hours before being examined under a microscope and photographed.

Study Design – Monolayer Cultures for Trypan Blue Staining

First passage chondrocytes from the MCP and MTP joints of a pony were grown to near confluence in 12-well plates. 37°C and 5% CO_2. Frozen stock solutions of curcumin (100mM) that had been previously prepared in DMSO, were defrosted and diluted in DMEM to 1mM. From this 1mM stock, test concentrations of curcumin (6μM, 12μM, 25μM, 50μM and 100μM) were prepared in DMEM and added to the appropriate wells. DMSO concentrations equivalent to those found in the curcumin 6μM treatment and 100μM treatment were added as vehicle controls. The plates were incubated with the treatments for 24 hours at 37°C and 5% CO_2 before adding 0.4% trypan blue solution (100μl) to the 1ml medium in each well. Non-viable cells absorb the trypan blue dye and stain blue, whereas viable cells with intact membranes exclude the dye. The plates were incubated for 5-10 minutes at room temperature, before removing the medium, placing on a haemocytometer and looking for stained cells under a microscope. PBS (1ml) was added to the adhered cells in the wells, before observing under a microscope. Five different views of cells were photographed per well.

Study Design – Monolayer Cultures for FACS analysis

First passage primary equine chondrocytes from the MCP and MTP joints of the pony used in the trypan blue study were grown to near confluence in 25cm^2 flasks. Culture medium was removed and replaced with either control medium (as previously described), 70% methanol to induce cell death, or curcumin at 3μM, 6μM, 12μM, 25μM, 50μM, or 100μM. Cells were placed in an incubator for 24 hours.

The following day, an Annexin V- fluorescein isothiocyanate (FITC) apoptosis detection kit (Sigma-Aldrich) was used according to the manufacturer's instructions. The reagents were equilibrated to room temperature before use. The culture medium was removed from each well into separate microcentrifuge tubes. The media were centrifuged at 9.6rcf for 10 seconds at room temperature and the resulting pellet resuspended in 500µl DMEM + FBS. The adhered cells were washed in the wells with PBS before trypsinising for 5 minutes. The trypsinised cells were placed into separate microcentrifuge tubes, centrifuged at 9.6rcf for 10 seconds at room temperature, and the resultant pellet resuspended in 500µl DMEM + FBS. Resuspended cells from the wells and the media were combined in corresponding microcentrifuge tubes, centrifuged at 9.6rcf for 10 seconds at room temperature, and the resulting pellets resuspended in 500µl 1X binding buffer. 500µl of cell suspension was placed into separate FACS tubes before adding 5µl annexin V-FITC conjugate to each cell suspension except unstained controls. 10µl propidium iodide (PI) was then added to each cell suspension and the tubes were incubated at room temperature for 10 minutes in the dark. The FACS machine (FACSCanto II, BD Biosciences) cycle was started and the results analysed using FACSDiva Software (version 6.1.2, BD Biosciences).

The FACS results are produced as a graph, and the locations of the cell populations within the graph can be interpreted as shown in figure 23. The percentage of cells in each quadrant were tabulated and graphed.

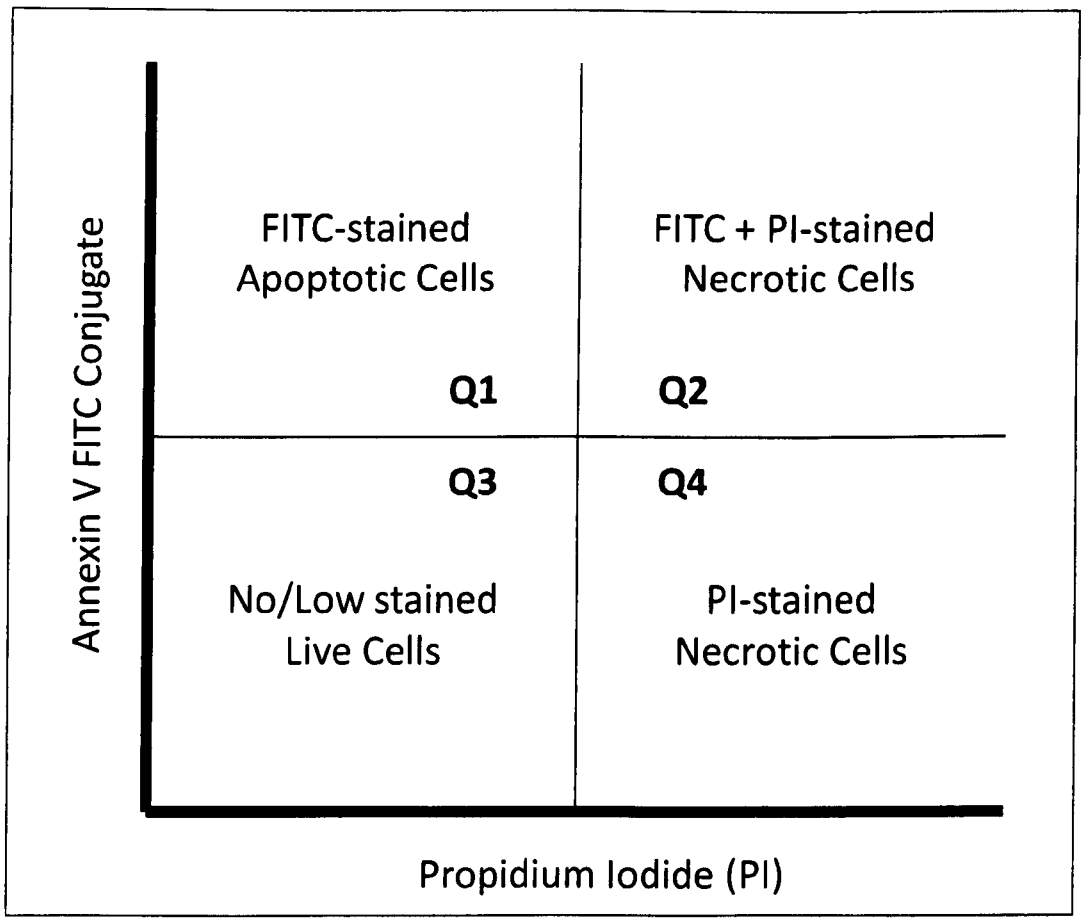

Figure 23. Key to cell populations in the FACS graphs

Annexin V is conjugated to the fluorescent dye FITC to facilitate the detection of bound annexin V by flow cytometry. Annexin V has a high affinity for phosphatidylserine, which translocates from the inner to the outside of the plasma membrane during apoptosis. Thus, apoptotic cells appear in quadrant (Q)1 of the graph. Propidium iodide (PI) stains deoxyribonucleic acid (DNA) and is impermeable to live cells, so as necrotic cells swell and burst their nuclear material stains positively for PI in Q4. However, necrotic cells also release phosphatidylserine when they burst, so they stain positively for both annexin V and PI and appear in Q2. Live cells do not stain with either annexin V or PI as they have intact surface membranes, so they are shown in Q3.

Study design – Monolayer Cultures for Cytotoxicity Assays

First and second passage primary equine chondrocytes from three horses and two ponies were grown to near confluence in 12-well plates. Culture medium was removed and replaced with treatment medium (1ml/well). Control wells contained medium alone (low glucose DMEM with 2% penicillin/streptomycin and 10% FBS) which formed the base for the other treatments. The NSAID carprofen (100μg/ml) was included as a positive control, as it had been used in previous studies. The NO donor, sodium nitroprusside (SNP; Sigma-Aldrich) was dissolved in DMEM (50mM) and used to induce cell death. Frozen stock solutions of curcumin (100mM), previously prepared in DMSO, were defrosted and diluted in DMEM to 1mM. From this 1mM stock, test concentrations of curcumin (3μM, 6μM, 12μM, 25μM, 50μM and 100μM) were prepared in DMEM and added to the appropriate wells. A DMSO control was prepared in DMEM and contained an equivalent volume of DMSO to that found in the highest curcumin concentration. The DMSO control was included on each plate to ensure that any observed effects were not due to the carrier solvent. The plates were incubated at 37°C and 5% CO_2. Toxicity was assessed after 24 hours, 48 hours and five days. Five day plates underwent a medium change mid-way through the experiment. The removed media were spun down and the resulting pellet of cells resuspended in fresh media containing curcumin before adding back to the appropriate wells.

Chondrocyte viability was assessed using a commercially available live/dead assay (Invitrogen) that utilises calcein AM and ethidium homodimer-1 to identify live and dead cells, respectively. The principle behind this method is that calcein AM is taken up by live cells and converted by intracellular esterases to fluorescent green

calcein. Ethidium homodimer-1 is impermeable to live cells but enters through the disrupted membranes of dead cells and stains the nucleic acids in their nuclei red.

Media were removed and centrifuged at 10,000rpm for 25 seconds at room temperature. The resulting pellet of detached cells (if any) was washed and resuspended in 20µl PBS. Adherent cells in the wells were washed in PBS before returning the whole detached cell suspension to the appropriate well and incubating in calcein AM (2µM) and ethidium homodimer-1 (4µM) in PBS for 30 minutes at room temperature. Fluorescence was detected and captured using an inverted contrasting microscope (Leica DM IL, Leica Microsystems Ltd) with Leica Application Suite imaging software (Version 2.4.0 R1, Leica Microsystems Ltd.). Six different fields of view of live and dead cells were taken per well (magnification x100). Live and dead cells were counted with Image J Software and the percentage of dead cells (expressed as a percentage of the total number of cells counted) was calculated at 24 hours, 48 hours and five days for each treatment.

Cartilage Explant Culture

Full depth cartilage shavings from the MCP joints of three Thoroughbred-type horses were cut into 3mm discs. Three discs per well from the same animal were placed in 24-well plates containing 1ml of culture medium (serum-free low glucose DMEM supplemented with 2% penicillin/streptomycin) and allowed to equilibrate overnight at 37°C under 5% CO_2. The following day, the culture medium was replaced with fresh medium before the study began.

Study Design – Cartilage Explant Model

All plates contained 1ml culture medium which formed the base for other treatments and acted as a control for each plate. Curcumin (12μM, 25μM and 100μM) was prepared in DMEM and added wells in quadruplicate. SNP (50mM) was used as a positive control for cell death. One well per treatment per animal was used for the explant cytotoxicity assay and the remaining three wells per treatment per animal were used to determine GAG release from unstimulated explants in response to curcumin. DMSO controls were performed previously and found to have no effect on GAG release from cartilage explants at volumes equivalent to that found in the highest curcumin concentration (data shown in chapter 2).

Plates were incubated at 37°C and 5% CO_2 for five days. After five days, explants were immediately assayed for cytotoxicity or frozen at -20°C with their corresponding supernatants for subsequent GAG assays as described on page 53.

Cartilage Explant Cytotoxicity Assays

After a five day incubation, chondrocyte viability was assessed using the live/dead assay. Explants were washed in PBS then incubated with calcein AM (2μM) and ethidium homodimer-1 (8μM) in PBS for 30 minutes at room temperature. A microscope slide was adapted to enable an explant to be studied under the confocal microscope without drying out. This was done using ring reinforcers to create a well for the explant to sit in (figure 24). The explant was covered with sterile PBS to prevent dessication. A cover slip was then placed over the explant fixing it in place. A confocal microscope (Leica TC SP2) was used to detect and measure fluorescence in 15μm z-sections through each explant (magnification x10).

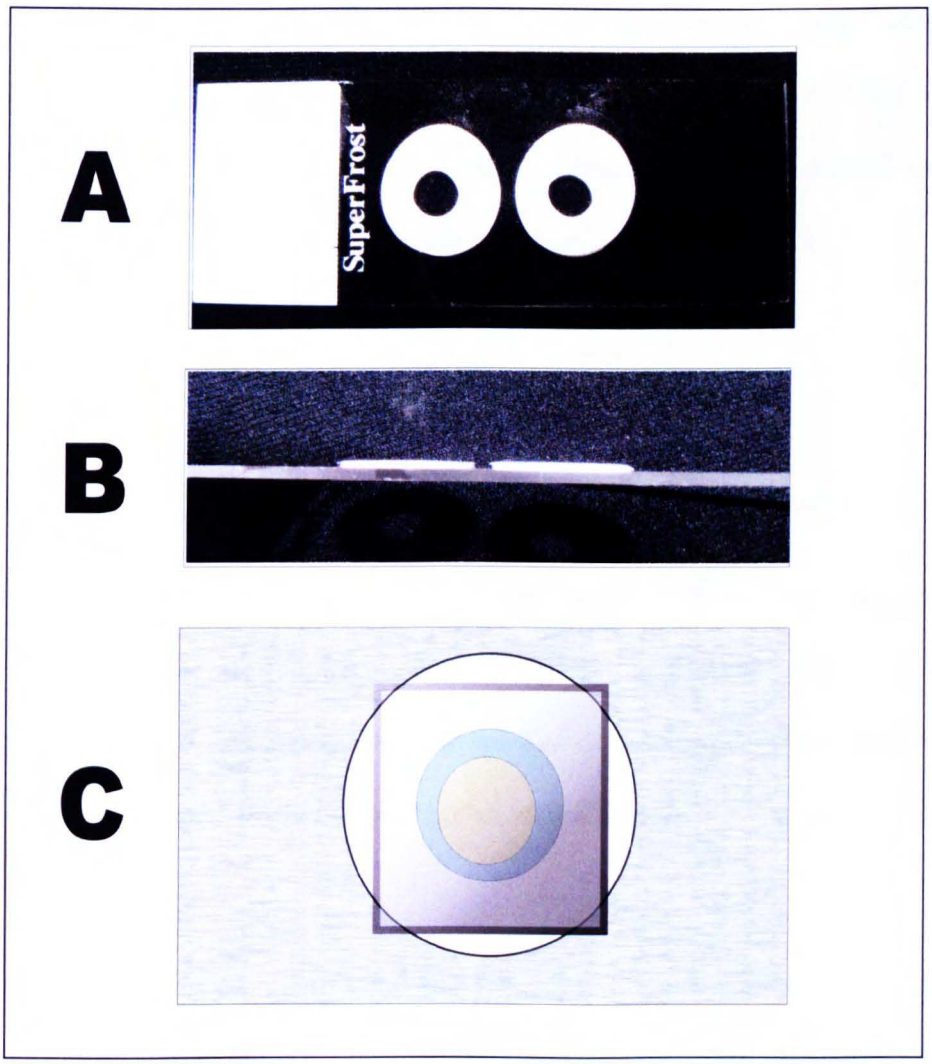

Figure 24. Slide adaptation for confocal imaging of cartilage explants

Photographs of the adapted slide with ring reinforcers from above (A) and from the side (B). C shows a diagram of the slide from above with the cartilage explant (yellow circle) in PBS (blue) within the ring reinforcer well, held in place by a square coverslip.

Statistical Analysis

Statistical analysis was performed on the percentage dead cell data from the cytotoxicity assays on combined animals for the monolayer cultures, using a one-way ANOVA with Tukey's *post hoc* test. Cytotoxicity was defined as the percentage of dead cells being significantly more ($p<0.05$) than controls.

3.3. Results

Curcumin (≥50μM) Alters Chondrocyte Morphology After 24 Hours

After 24 hour incubation with curcumin at concentrations of 50μM and over, primary equine chondrocyte morphology was markedly different from controls. Chondrocytes became more spherical in shape and detached from the surface of the culture plate. Methanol-treated chondrocytes stayed attached to the surface (figure 25).

At a higher magnification, control and most curcumin (25μM)-treated chondrocytes stayed attached to the culture plate in their monolayers after 24 hours. Some chondrocytes cultured with 25μM curcumin showed signs of starting to become more spherical but did not detach. Twenty-four hour culture with curcumin (50μM and above) caused chondrocytes to become more spherical in shape and detach from the surface of the culture plate. Methanol-treated chondrocytes stayed attached to the surface, but were less defined and appeared to have some holes on the surface of the cells (figure 26).

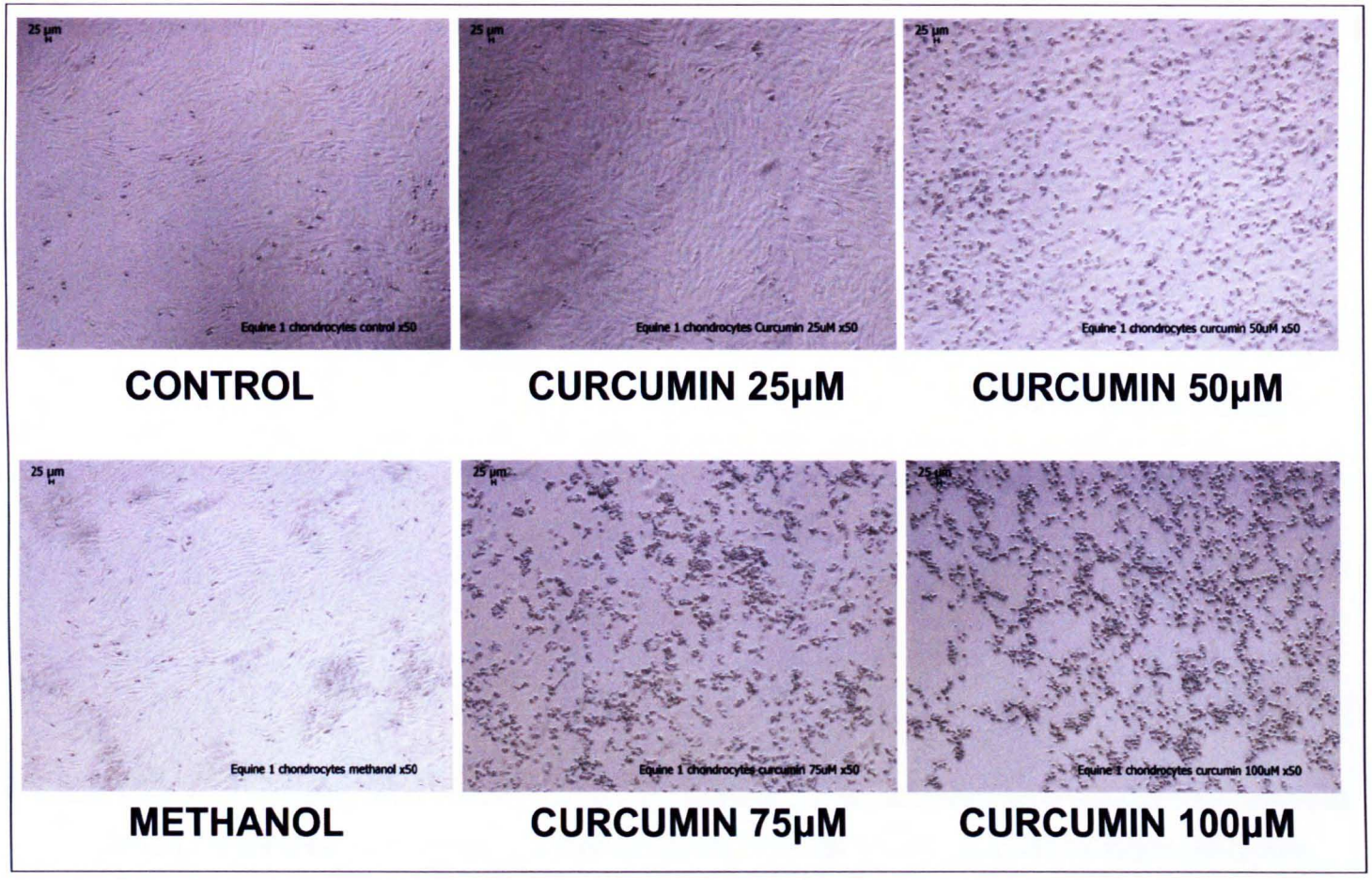

Figure 25. Phase contrast photomicrographs of third passage primary equine chondrocytes after 24 hour culture in serum supplemented medium (control), methanol (positive dead control) and curcumin (25-100µM), magnification x50

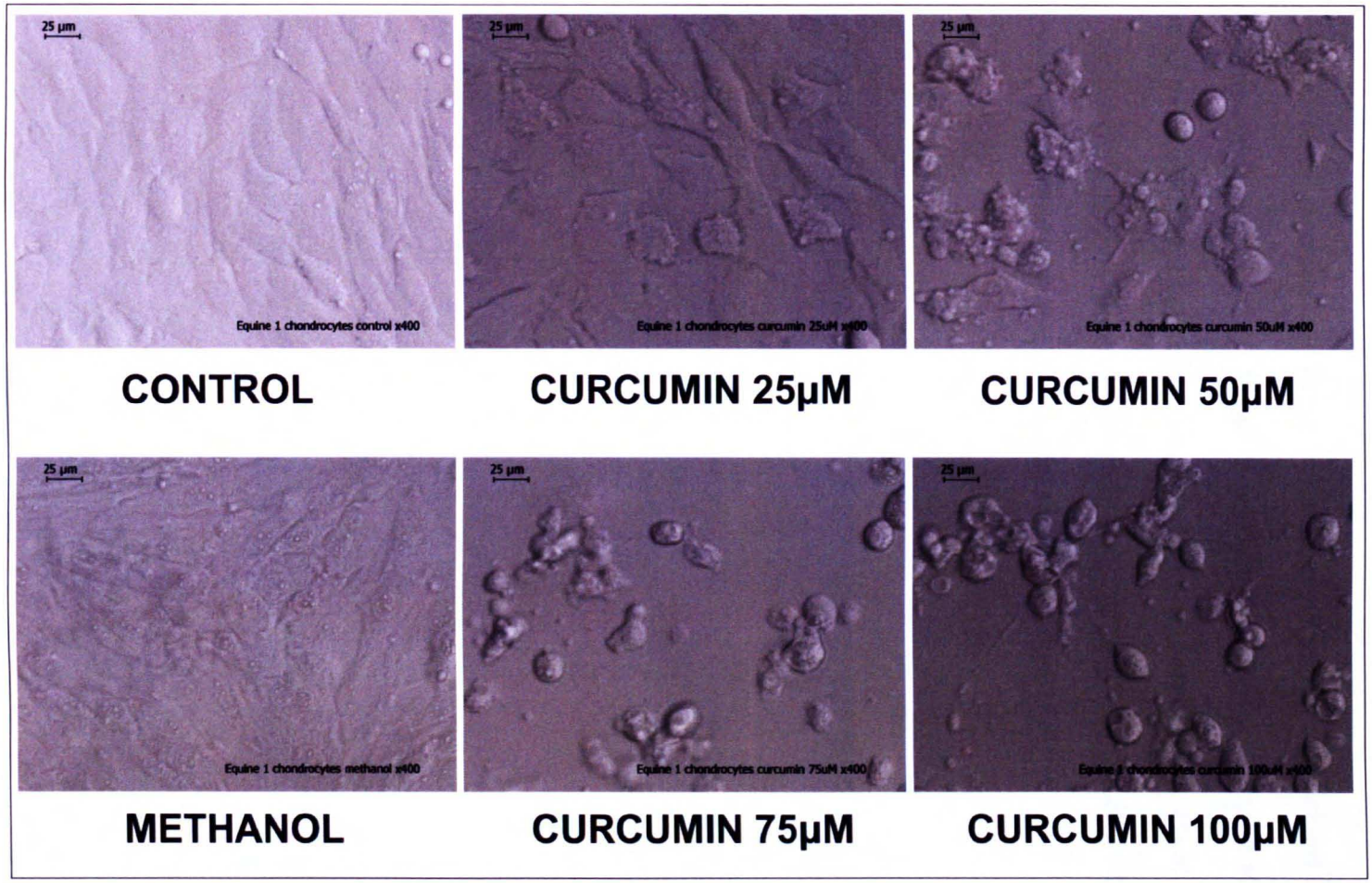

Figure 26. Phase contrast photomicrographs of third passage primary equine chondrocytes after 24 hour culture in serum supplemented medium (control), methanol (positive dead control) and curcumin (25-100µM), magnification x400

Increasing Curcumin Concentrations Increase Cell Death – Trypan Blue Staining

The change in morphology with increasing curcumin concentrations was accompanied by increased cell death, as indicated by trypan blue staining in cells at curcumin concentrations of 25μM and over (figure 27). However, in this study, cells started to detach with 12μM curcumin; an effect not observed with 25μM curcumin in the previous study. It must be noted here that it was difficult to distinguish between stained and unstained cells under the microscope.

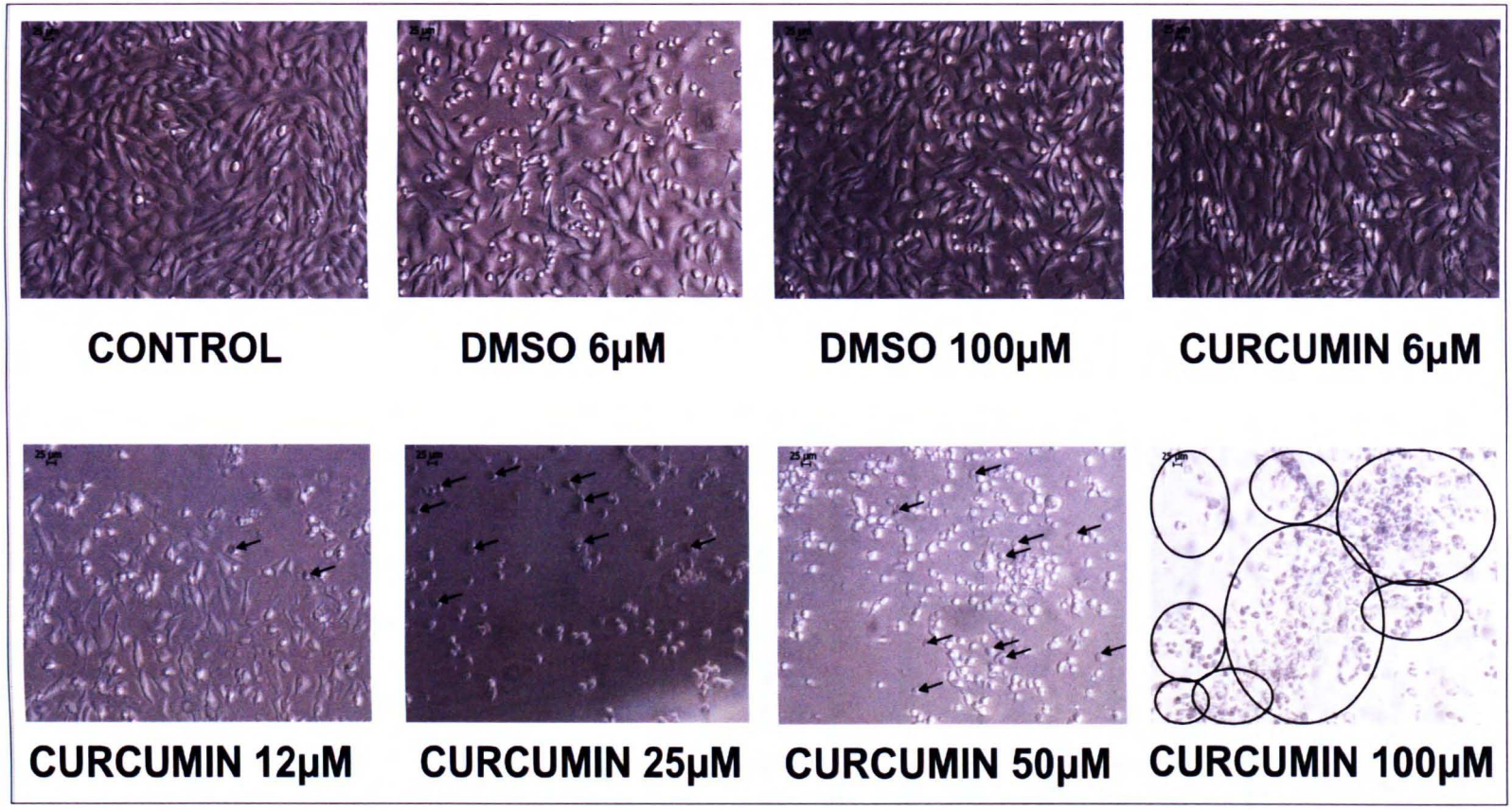

Figure 27. Trypan blue stained first passage primary equine chondrocytes after 24 hour culture in serum supplemented medium alone (control), with dimethyl sulfoxide (DMSO) or with curcumin (6-100µM), magnification x100

DMSO concentrations were equivalent to that in curcumin 6µM (DMSO low) and 100µM (DMSO high) treatments. Trypan blue positive cells are denoted by arrows or circles encapsulating groups of cells.

Increasing Curcumin Concentrations Increase Cell Death – FACS

The unstained controls defined the cell population from which the remaining cells were determined and showed that there was low autofluorescence in the sample population (Table 2 and Figure 28). Stained controls were used to confirm the viability of the population with minimal staining for both PI and Annexin V. However, the stained control showed that only half of the population was viable with 64.9% live chondrocytes, and a large proportion of necrotic cells. The methanol dead control had 14.9% live chondrocytes. Compared to controls, curcumin 3μM, 6μM and 12μM had good viability with 74.9%, 79.6 and 77.9% live cells respectively. However, as the concentration of curcumin increased the cell population moved towards the upper right hand quadrant (necrotic cell death) and cell viability decreased with the percentage of live cells falling to 6.2% (curcumin 25μM). This was more pronounced at higher concentrations with 3.1 % live cells (50μM) and 0.1% (100μM).

Quadrant (Q)	Percentage of cells								
	Control U	Control S	Control M	Curcumin					
				3μM	6μM	12μM	25μM	50μM	100μM
Q1 – (FITC) Apoptotic	0.0	4.9	2.8	3.5	2.1	7.6	8.8	2.9	0.0
Q2 – (FITC+PI) Necrotic	0.2	28.0	36.9	16.8	10.0	12.6	84.7	94.0	99.8
Q3 – (Unstained) Live	99.7	64.9	14.9	74.9	79.6	77.9	6.2	3.1	0.1
Q4 – (PI) Necrotic	0.0	2.2	45.4	4.8	8.3	1.9	0.3	0.1	0.0
Q2 + Q4 - Total Necrotic	0.2	30.2	82.3	21.6	18.3	14.5	85.0	94.1	99.8

Table 2. Percentages of primary equine chondrocyte cells in their first passage cultured with curcumin (3-100μM) in each quadrant on the FACS graphs

Percentage of cells stained with Annexin V-FITC conjugate and/ or Propidium Iodide, or neither. Stained (S) and unstained (U) controls represent media from explants cultured in culture medium alone. Methanol (M) control represents the dead control.

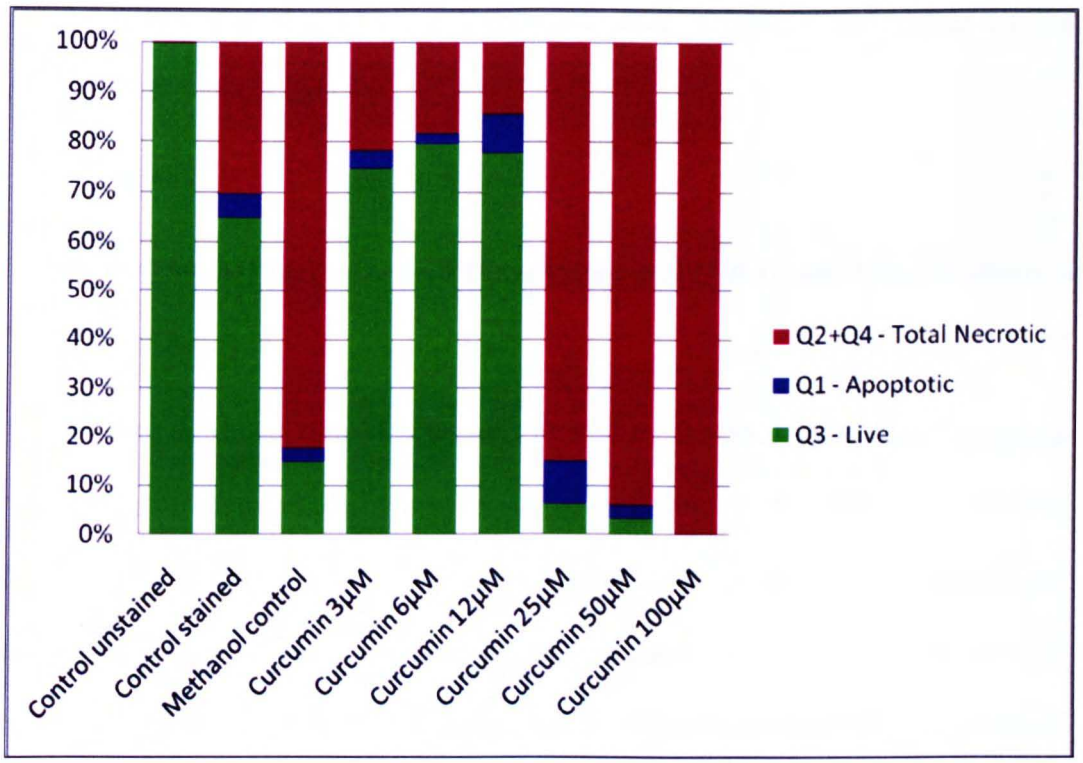

Figure 28. Graph to show the percentage of live, apoptotic and necrotic cells from the FACS results of primary equine chondrocytes in their first passage cultured with curcumin (3-100µM)

Percentage of cells stained with Annexin V-FITC conjugate (Q1 – Apoptotic) and/ or Propidium Iodide (Q2+Q4 – Total Necrotic), or neither (Q3 – Live). Stained and unstained controls represent media from explants cultured in culture medium alone. Methanol control represents the dead control.

Despite this indication of curcumin toxicity towards chondrocytes, the large quantity of dead cells in the stained control, means that no definitive conclusions can be drawn from the FACS data.

Curcumin (≥50μM) Increases Cell Detachment and Cell Death After 24 Hours – Cytotoxicity Assay

Curcumin at 50μM and 100μM noticeably altered the morphology of adherent equine articular chondrocytes at the light microscopic level (figure 29) compared to control. Chondrocytes became more spherical in the presence of curcumin (≥50μM) and detached from the surface of the plate but this change was not present with the same concentration of DMSO alone (the agent used to solubilize curcumin). This altered morphology was visible in a few cells at 25μM curcumin but at 50μM the majority of cells were affected.

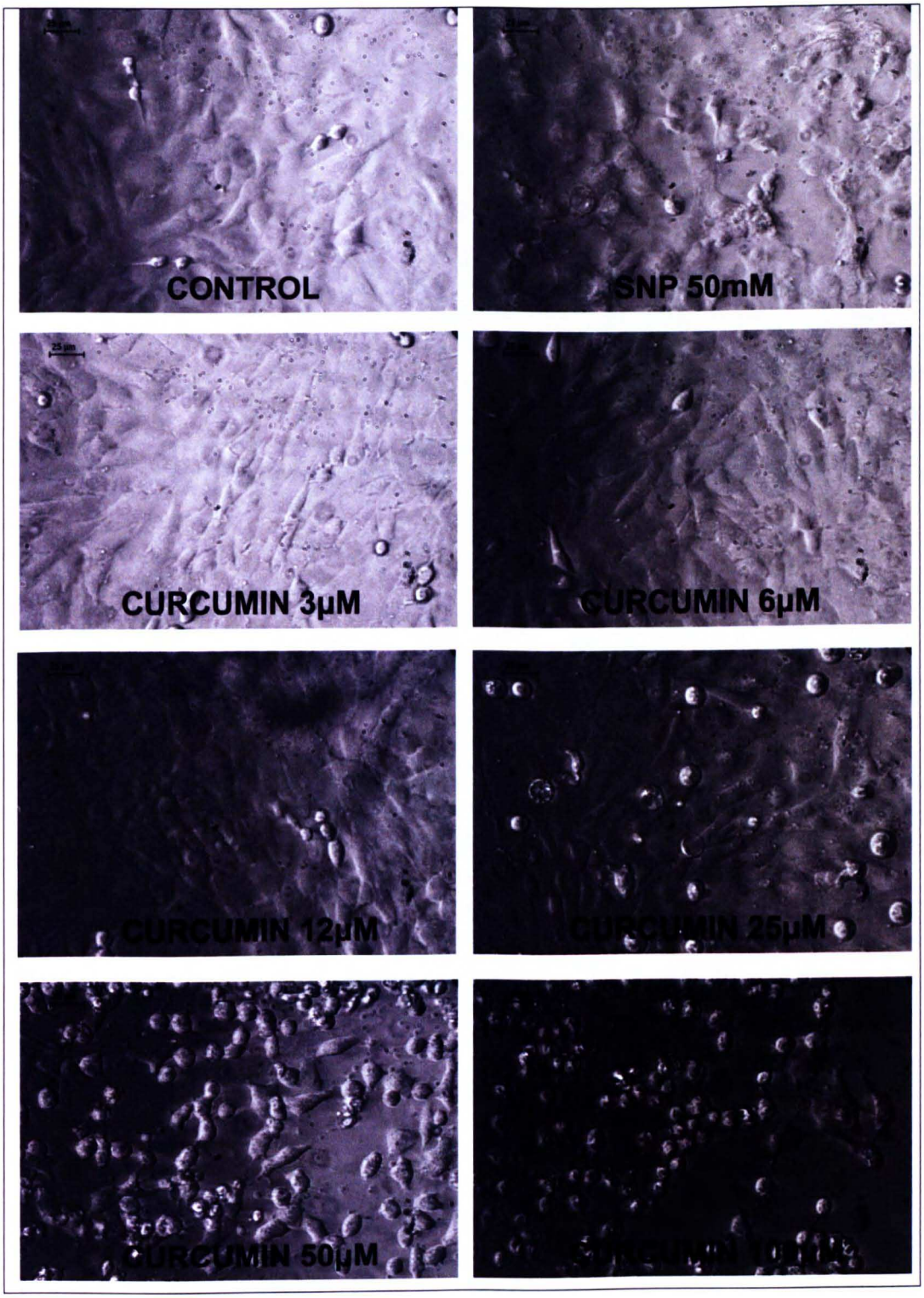

Figure 29. Phase contrast photomicrographs of second passage primary equine chondrocytes after 24 hour culture in serum supplemented medium (control), sodium nitroprusside (SNP; 50mM) and curcumin (3-100µM), magnification x400

The morphological changes induced by curcumin 50μM were associated with an increase in cell death as indicated by less green (live)-stained cells and more red (dead)-stained cells (figure 30). Neither control nor curcumin at concentrations of 25μM and below had any apparent effect on chondrocyte viability.

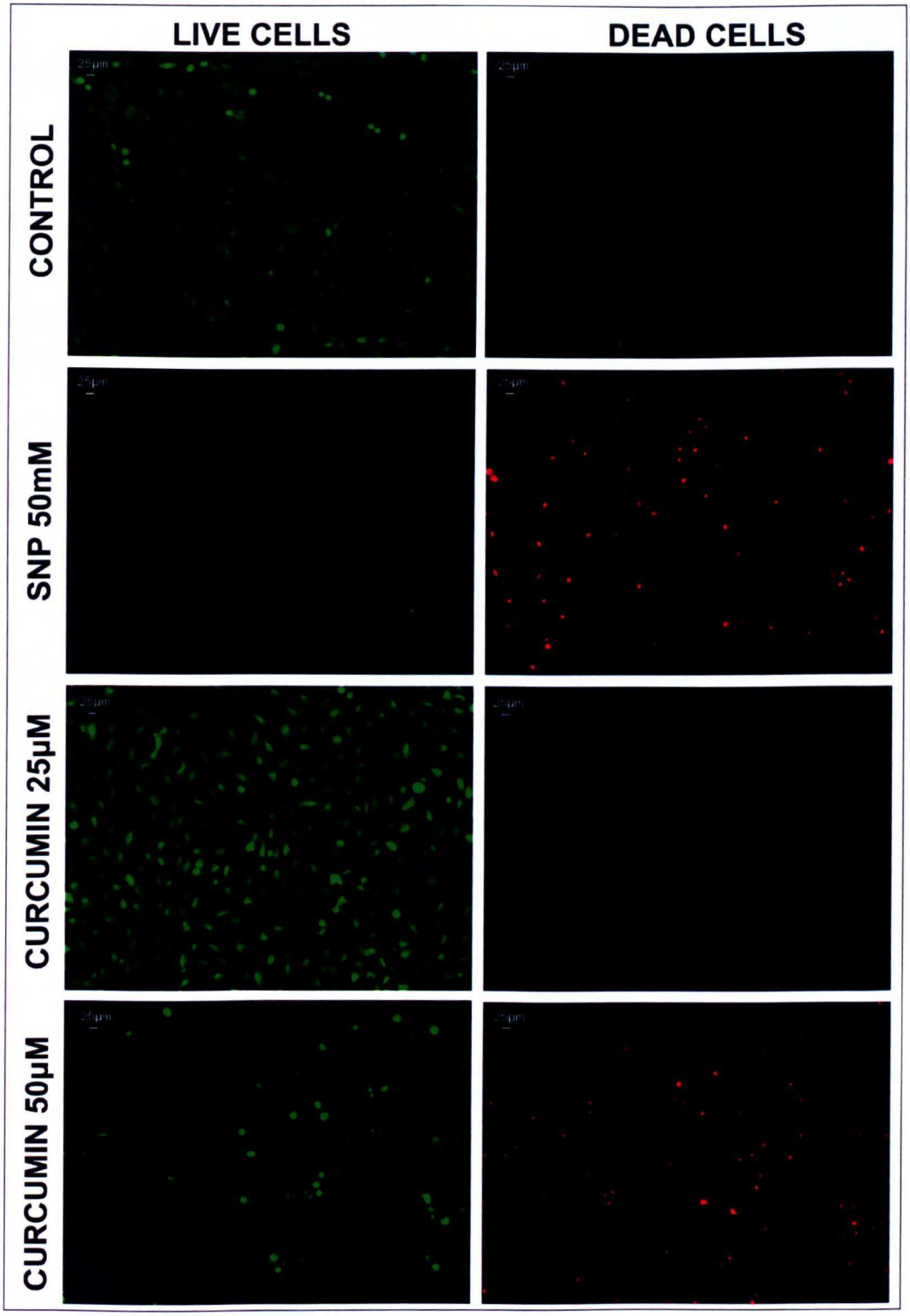

Figure 30. Fluorescent inverted contrast microscope images of live (green) and dead (red) second passage primary equine chondrocytes after 24 hour culture in serum supplemented medium (control), sodium nitroprusside (SNP; 50mM), and curcumin (25µM, 50µM), magnification x100

The numbers of dead cells were counted in each random field of view under the microscope for each horse. Significance levels were the same for all horses. Figure 31 shows the results from the young Thoroughbred type horse chondrocytes (morphology and live/dead staining shown in figures 29 and 30). Statistical analysis revealed that control chondrocytes had mean cell death of 0.72 ± 0.16% at 24 hours. DMSO has no significant effect on cell death at concentrations equivalent to 3μM and 100μM curcumin. SNP significantly increased cell death ($p<0.001$) with mean cell death of 100 ± 0.0% at 24 hours. Curcumin had no significant effect on mean cell death at concentrations of 3μM (0.66 ± 0.30%), 6μM (0.61 ± 0.19%), 12μM (1.56 ± 0.35%), 25μM (2.56 ± 0.60%) after 24 hours. However, curcumin significantly increased cell death at 50μM (81.13 ± 5.15, $p<0.001$) and 100μM (99.42 ± 0.37, $p<0.001$).

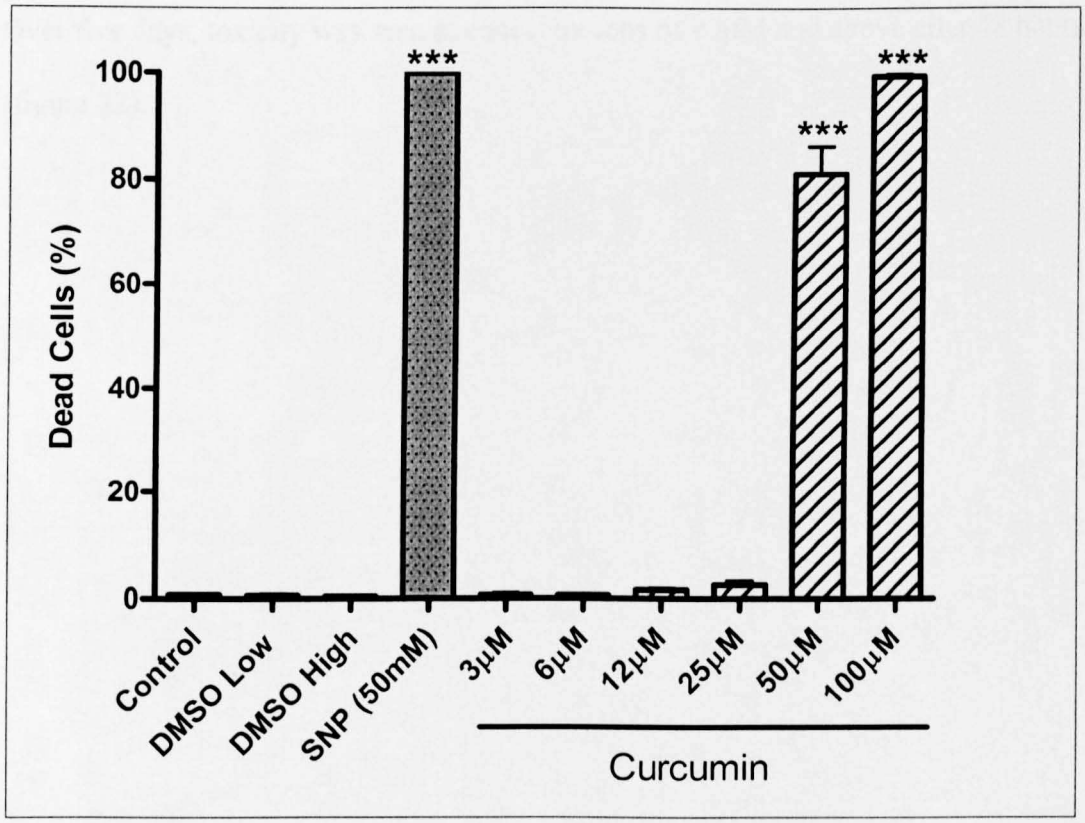

Figure 31. Percentage dead chondrocyte cells from the total number counted after 24 hour culture with curcumin (3-100μM)

*Second passage primary equine chondrocytes were cultured in media alone (control), in dimethyl sulfoxide (DMSO) at concentrations equivalent to those found in the curcumin 3μM (DMSO Low) and curcumin 100μM (DMSO High) wells. Results are expressed as the mean percentage of dead cells per field of view per treatment and bars represent standard error of the means (SEM). Significance compared to control is indicated by *** ($p<0.001$).*

Curcumin (≥25μM) Increases Cell Death After 48 Hours – Cytotoxicity Assay

Over five days, toxicity was seen at concentrations of 25μM and above after 48 hours (figure 32).

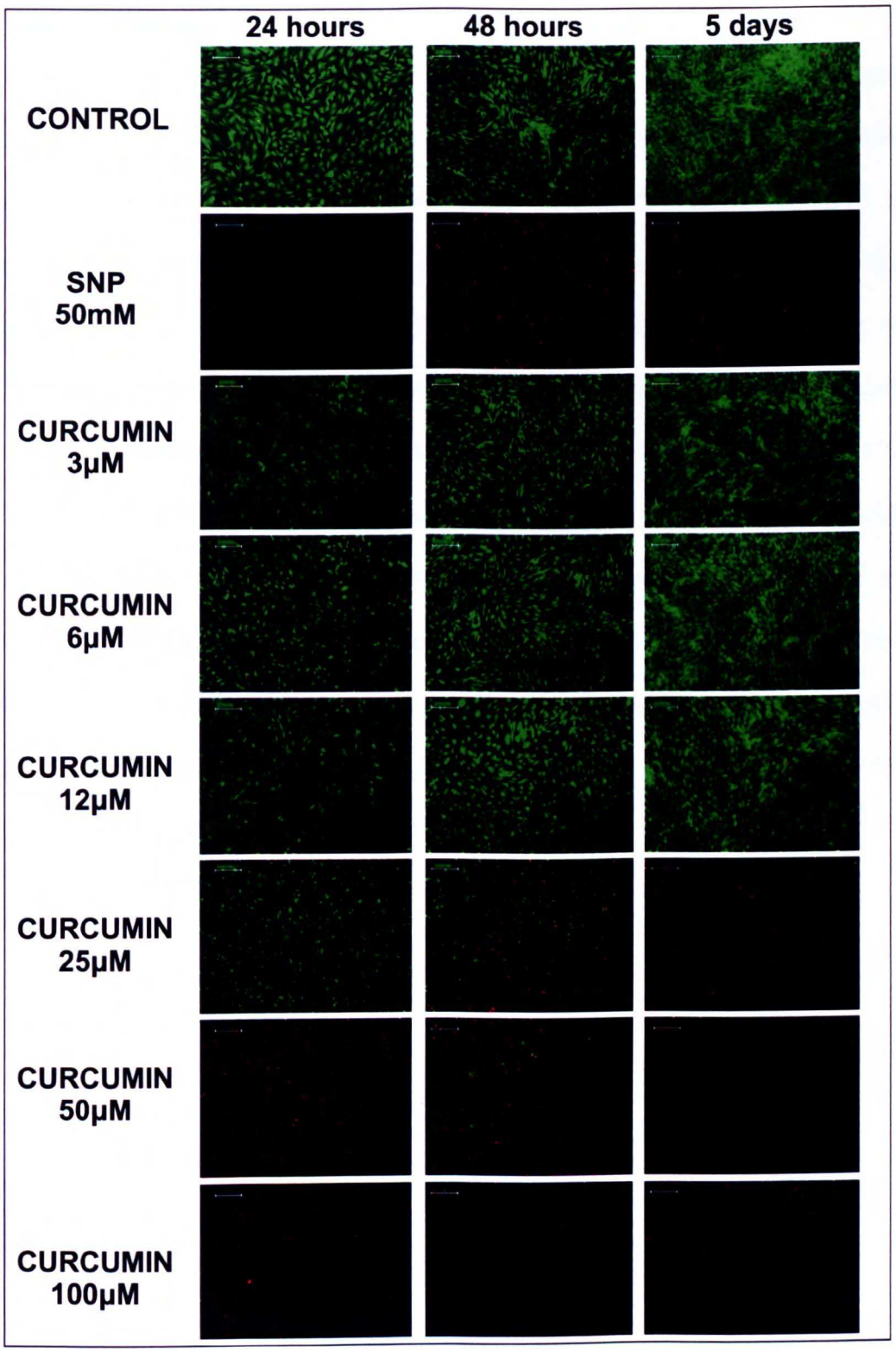

Figure 32. Fluorescent images of live (green) and dead (red) first passage primary equine chondrocytes after 24 hour, 48 hour and 5 day culture in serum supplemented medium (control), sodium nitroprusside (SNP; 50mM) and curcumin (3-100μM), magnification x100, scale bars = 200μm

Quantification of dead cells expressed as a percentage of the total number of cells counted from all horses showed that untreated controls had a mean cell death percentage of less than 1% at 24 hours, 48 hours and five days (figure 33). DMSO controls and the NSAID did not significantly increase cell death compared to controls at all time points. SNP effectively induced cell death ($p<0.001$ at all time points) with a mean cell death of 92.95 ± 2.29% at 24 hours, 99.6 ± 0.17% at 48 hours and 100 ± 0.00% at five days. Curcumin significantly increased cell death compared to controls after 24 hours at concentrations of 50μM (71.75 ± 7.25%, $p<0.001$) and 100μM (99.55 ± 0.12%, $p<0.001$). After 48 hours a significant increase in toxicity compared to controls was seen at concentrations of 25μM (30.67 ± 8.94%, $p<0.001$), 50μM (95.9 ± 0.96%, $p<0.001$) and 100μM (99.59 ± 0.18%, $p<0.001$). After five days in culture, curcumin at 25μM caused a significant increase in cell death compared to controls (95.71 ± 0.72%, $p<0.001$), 50μM (99.4 ± 0.39%, $p<0.001$) and 100μM (100 ± 0.00%, $p<0.001$).

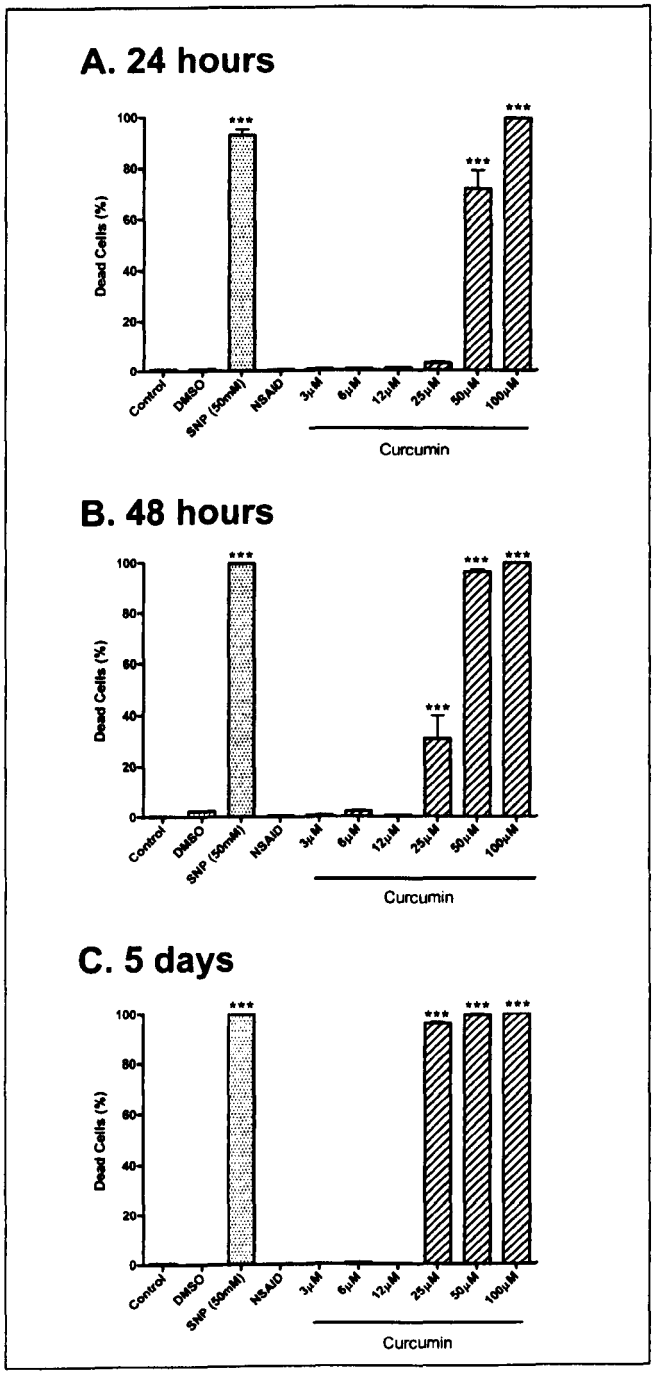

Figure 33. Percentage chondrocyte cell death from the total counted after 24 hours (A), 48 hours (B) and 5 days (C) with curcumin (3-100μM)

*Cells cultured in media alone (control), or with dimethyl sulfoxide (DMSO) equivalent to that in the curcumin 100μM treatment (vehicle control), sodium nitroprusside (SNP; 50mM) or curcumin. Results are expressed as the mean percentage of dead cells per field of view per treatment and bars represent standard error of the means (SEM). Significance compared to control is indicated by *** ($p<0.001$).*

Curcumin (25μM) is Not Cytotoxic to Equine Cartilage Explant Chondrocytes After 5 Days

After five day culture without serum supplementation, explants retained fully viable chondrocytes as indicated by the intensive green staining and lack of red staining in the controls (figure 34). SNP induced cell death in the explants as shown by the increased red staining and fewer, less vibrant green stained cells in comparison to the controls. Curcumin (12μM and 25μM)-treated explants contained large numbers of green stained cells, suggesting that neither concentration was detrimental to the viability of chondrocytes after five days in explant culture. However, the large amount of red nuclei staining to the chondrocytes in the 100μM curcumin-treated explants indicates that curcumin was highly cytotoxic at this concentration.

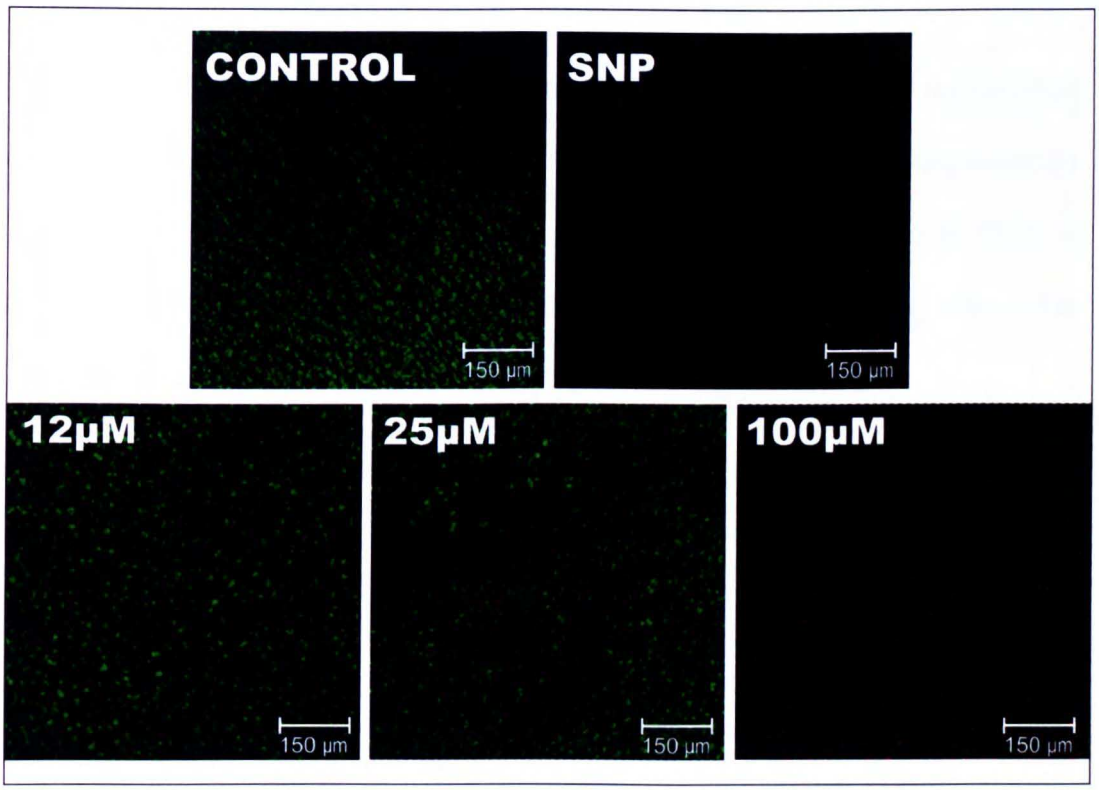

Figure 34. Overlaid images of 15µm z-sections through cartilage explants after 5 days in culture with curcumin (12-100µM)

Control consists of culture media with 2% penicillin/streptomycin. Treatments consist of sodium nitroprusside (SNP; 50mM), or curcumin (12µM, 25µM and 100µM) in culture media. Green staining indicates live metabolizing cells and red staining highlights the nuclei of dead cells, magnification x10.

Curcumin (12-100μM) has No Effect on GAG Release From Unstimulated Equine Cartilage Explants

Control explants released 13.91 ± 1.13% of the total GAG content of the cartilage into the medium over five days (range: 159-414μg GAG/ml of culture supernatant) (figure 35). SNP (50mM) significantly increased matrix GAG release to 82.01 ± 2.43% ($p<0.001$) after the same period. Curcumin did not significantly alter GAG release from the explants after five days at 12μM, 25μM and 100μM.

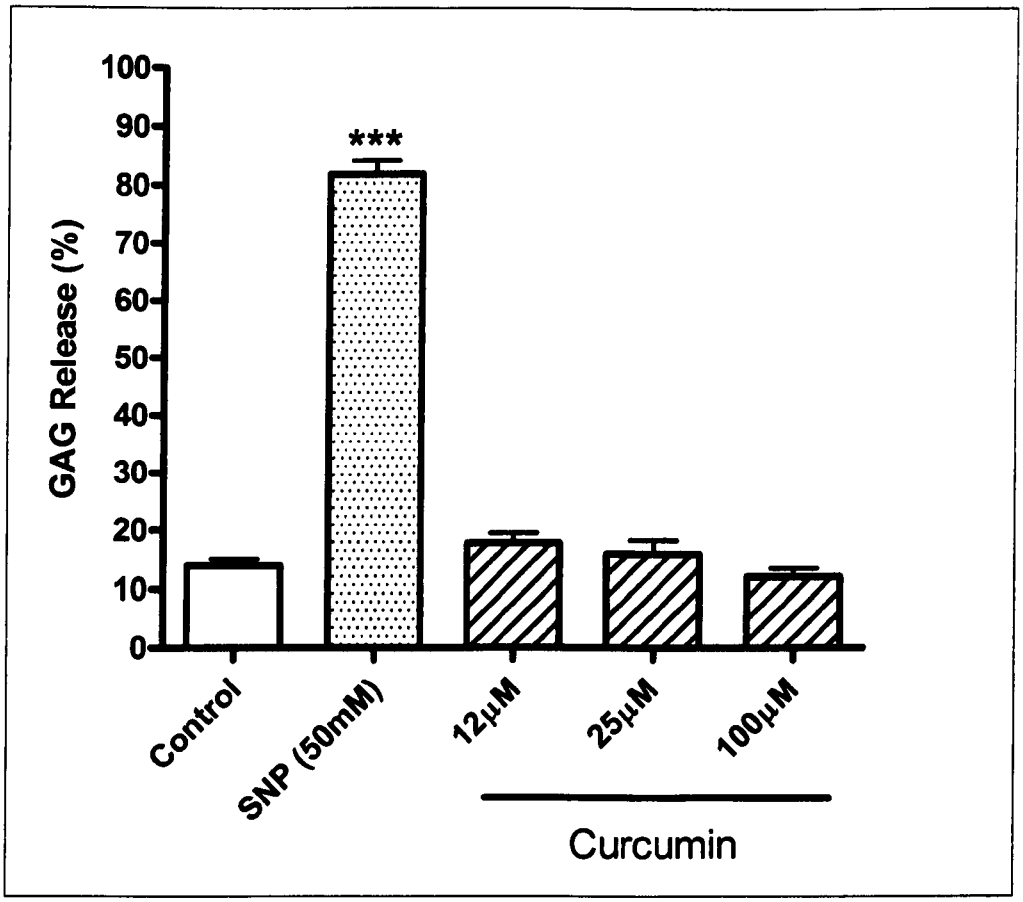

Figure 35. Effect of curcumin (12-100μM) on percentage glycosaminoglycan (GAG) release from unstimulated cartilage explants

*Control column indicates cartilage discs incubated in the culture medium alone and sodium nitroprusside (SNP; 50mM) was used as a positive control for cell death. Values are reported as the mean of 3 horses per treatment and bars represent standard error of the means (SEM). Significance compared to control is indicated by *** ($p<0.001$).*

3.4. Discussion

The previous chapter in this thesis showed that curcumin has anti-catabolic and anti-inflammatory effects on IL-1β-stimulated cartilage at concentrations from 3-100μM. However, recent research suggests that curcumin (50μM) is cytotoxic to a chondrocyte cell line *in vitro* (Toegel *et al.*, 2008). This study addressed the issue of curcumin cytotoxicity in equine cartilage explants and monolayer chondrocyte cultures. Phase contrast microscopy showed that after 24 hour culture with curcumin (50μM and above) monolayer chondrocytes began to round and detach from the wells. Thereby suggesting that the cells were losing their membrane anchor proteins, such as integrins, which are involved in cell attachment and survival (Bates *et al.*, 1995). A variety of methods were employed to detect whether curcumin was inducing cytotoxicity, including trypan blue, flow cytometry and live/ dead viability assay using two-colour fluorescence. Although the trypan blue method is a well-established method of determining cell viability, it has been reported to overestimate cell viability (Altman *et al.*, 1993). In agreement with this, it was difficult to distinguish between stained and unstained cells under the microscope and resulted in inaccurate estimations of viability. In addition, the trypan blue method was time-consuming, inconsistent, and was limited by only confirming cell death not viability.

The results from the FACS method indicated that increasing concentrations of curcumin increased necrotic cell death of the chondrocytes. This is in contrast to the published literature showing that curcumin induces cell death through apoptosis in both cancer cells (Jiang *et al.*, 1996) and non-tumour cells such as neutrophils (Jackson *et al.*, 2006). However, other studies have suggested that curcumin caused apoptosis up to 50μM, but necrosis at 100μM in two breast tumour cell lines and a hepatocellular carcinoma cell line (Syng-Ai *et al.*, 2004). Again, in contrast to this, a

recent study using FACS analysis on pituitary tumour cells reported a time dependent increase in the proportion of necrotic cells up until 12 hours of culture with curcumin (30μM), but this was always accompanied by a larger increase in apoptotic cells, the proportion of which continued to increase after 24 hours (Schaaf *et al.*, 2010). Admittedly, this may be different with primary cells, as work has suggested that curcumin concentrations up to 100μM are cytotoxic to tumour cell lines but show no detriment to viability of rat hepatocytes after 24 hours (Syng-Ai *et al.*, 2004). However, the data in this chapter show that curcumin (50μM and 100μM) significantly increased cell death in primary equine chondrocytes after 24 hours. Whether this was by an apoptotic or necrotic mechanism is still to be discovered as the increased necrotic cell death seen in the present study may be due to the purity of curcumin used. For example, Schaaf *et al.* (2010) used a 95% pure curcumin from Sigma-Aldrich, whereas the studies in this chapter used a 70% pure preparation. Consequently, the toxicity may have been due to impurities in the remaining 30% of the product. However, there was always a very high level of cell death in the control populations, suggesting that the process of preparing the cells for the FACS analysis was causing them to die by necrosis, e.g. trypsinisation. Therefore, the data were not considered reliable. Although the FACS method, if working correctly, would have distinguished between apoptosis and necrosis as the mode of cell death, this information was not essential for a screening method to detect general toxicity. In addition, the machine was expensive and unreliable to use, so the FACS method was not pursued further. However, the information on the mode of cell death would be a useful addition to future studies looking at a specific ingredient.

Consequently, the live/dead assay was selected as the method of detecting chondrocyte death as it can relatively quickly provide qualitative and quantitative

data on both monolayer and explant chondrocyte cultures (Aizawa *et al.*, 2001; Grogan *et al.*, 2002). Although this method has been reported to overestimate cell viability in osteochondral allografts, (Lightfoot *et al.*, 2007), the use of two fluorescent dyes clearly separated live cells from dead cells and thus reduced the risk of the false positive results found with the trypan blue method. It should be noted here that cell death in monolayer cells can be underestimated, as dead cells can detach and be removed with the medium (Puttonen *et al.*, 2008). However, this risk was minimized by centrifuging the discarded media so that the resulting pellet of detached cells could be resuspended and returned to the well before the live/dead stain reagents were added.

At the same time as the live/dead stain method was developed, the positive control for cell death was changed from methanol to SNP. Methanol was recommended by the manufacturer of the live/dead assay as a suitable method for killing cells. However, methanol appeared to fix the cells, supported by the fact that methanol has been used to fix chondrocyte cultures previously (Cheung *et al.*, 2001). In addition, methanol did not cause 100% death in the FACS assay. Thus, SNP was selected as an alternative inducer of cell death, due to its known cytotoxicity towards chondrocytes (Notoya *et al.*, 2000). SNP (50mM) was an effective positive control for cell death, causing 100% death of chondrocytes after 24 hours.

In contrast to the monolayer cell cultures, curcumin (25μM) did not show cytotoxicity towards chondrocytes cultured in cartilage explants after 5 days, although toxicity was observed at 100μM. Within cartilage, chondrocytes are embedded in a complex ECM across which diverse diffusion gradients and fluid flow occurs (Garcia *et al.*, 1996). This could account for the differing toxicity threshold between monolayers and explants. Chondrocytes are more vulnerable to cell death

when they are no longer encased within a matrix, as cell adhesion and integrin attachments are important factors in promoting cell survival (Cao *et al.*, 1999). Thus, without a protective ECM, monolayer chondrocytes may be more exposed and susceptible to external agents in the culture medium than those within explants, as has been shown with bupivacaine (Chu *et al.*, 2006).

The results of this investigation suggest that curcumin, at concentrations of 25μM, induces chondrocyte death in monolayer primary equine chondrocytes after 48 hours, but not in cartilage explants after five days. The cytotoxic effects of curcumin (50μM) have been observed in other non-equine cell types, notably tumour cell lines (Moon *et al.*, 2005). Concentrations as low as 8μM caused a maximal decline in cell viability of 77% in a Ewing sarcoma cell line (Singh *et al.*, 2009). In fact, the induction of apoptosis through cytochrome *c* release and subsequent caspase activation is thought to be a key chemopreventive effect of curcumin in cancer studies (Anto *et al.*, 2002; Woo *et al.*, 2003). Curcumin (50μM) has also been shown to reduce the viability of an immortalised human chondrocyte cell line after 24 hours (Toegel *et al.*, 2008). The results from this chapter suggest that this is also the case with primary equine chondrocytes. However, a recent study on primary human chondrocytes found that curcumin (50μM) did not reduce cell viability and successfully inhibited IL-1β-induced cytotoxicity as demonstrated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Csaki *et al.*, 2009). This could be due to species-specific differences in chondrocyte susceptibility to curcumin, differences between assays used to measure viability, or more likely, the different sources of curcumin used. This highlights the importance of assessing toxicity alongside efficacy, with the same form of curcumin in each individual study. In addition, it suggests that testing multiple batches of curcumin on

the cells would be beneficial to monitor batch variability. For comparative purposes, it would be useful to conduct further studies with other cell types, such as the transformed chondrocyte cell line as tested by Toegel *et al.* (2008) to evaluate the results in this chapter with those from other laboratories.

Once the uppermost observed safe level of curcumin was determined in monolayer primary chondrocytes, the effect of curcumin and SNP on cartilage explants was determined. No significant effect of curcumin alone on matrix GAG release from normal cartilage explants at both safe and toxic concentrations was observed. This suggests that curcumin does not alter the basal level of GAG released from unstimulated cartilage explants in culture, even at cytotoxic concentrations. Interestingly, in these unstimulated explants, the two treatments that caused cell death had different effects on GAG release: SNP (50mM) caused extensive GAG loss into the medium whereas curcumin (100μM) did not alter GAG release compared to controls. GAG loss does not directly lead to cell death in cartilage explants (Otsuki *et al.*, 2008), thus it can be considered that the GAG loss and cell death caused by SNP are likely to involve different mechanisms. SNP generates NO, ceramide and cyanide which, aside from causing apoptosis, reduce proteoglycan and collagen synthesis by chondrocytes and increase inflammatory mediator production and MMP activity (Blanco and Lotz, 1995; Murrell *et al.*, 1995; Khatib *et al.*, 2002) resulting in cartilage matrix loss. Conversely, although curcumin (100μM) induces cell death, it is likely to use a mechanism that does not involve the release of many proteases. Curcumin is known to reduce the release of inflammatory mediators, MMPs and NO (Brouet and Ohshima, 1995; Shakibaei *et al.*, 2007). The previous chapter showed that cytotoxic levels of curcumin reduced IL-1β-stimulated PGE_2 and MMP-3 release. This reduced production of inflammatory mediators and

catabolic enzymes, suggests that curcumin is unlikely to induce extensive GAG loss as demonstrated in both IL-1β-treated and untreated cartilage explants in this study.

In conclusion, curcumin at 25μM and below is not cytotoxic to cartilage explants after 5 days in culture, and curcumin at 12μM and below is not cytotoxic to monolayer primary equine chondrocytes after 5 days. The previous chapter showed that using the same batch of curcumin, concentrations as low as 3μM effectively reduced GAG and PGE_2 release from IL-1β-stimulated cartilage explants. Thus non-cytotoxic concentrations of curcumin effectively attenuate the catabolic and inflammatory effects of IL-1β in the model.

The results from this chapter only provide an indication of toxicity to a specific cell type, from a specific species in this particular culture system using a certain batch of curcumin. Conclusions cannot be drawn regarding the toxicity of curcumin to other cell types *in vitro* or even *in vivo*. However, though the results do not signify safety outside of this culture system, they do indicate that the positive effects of curcumin seen at lower concentrations *in vitro* are not a consequence of cell death. This highlights the importance of toxicity testing any potential substances for testing in the model. This chapter also established that the live/dead stain was a rapid and consistent method for detecting toxicity. A valuable finding from this research was that monolayer chondrocytes were more sensitive to curcumin in media and showed signs of cytotoxicity at lower concentrations than those embedded within cartilage explants. Thus, it was decided that future studies would use monolayer chondrocytes to assess toxicity of potential joint health products to determine concentrations suitable for testing in the explant model.

CHAPTER 4. *IN VITRO* ASSESSMENT OF A PLANT EXTRACT COCKTAIL

4.1. Introduction

The previous chapters set the scene for developing and testing the *in vitro* models and establishing the methods to determine cytotoxicity and efficacy, in terms of reducing GAG and PGE_2 release from IL-1β-stimulated cartilage explants. Once these were established, a cocktail of plant extracts (referred to as PT) was tested in the model. The rationale for this was that combinations of ingredients in a formulation are likely to be more potent as they may act synergistically to produce a greater overall effect (Williamson, 2001). For example, a product containing an anti-inflammatory ingredient and anti-catabolic ingredient may improve the overall efficacy of the product. Using a combination product also tested the ability of the model and the assays to detect effects of different ingredients in combination. In addition, the PT was intended for a field trial, so it was of interest to test it in the models first to assess its effects *in vitro*.

The PT ingredients included *Boswellia serrata* extract, grape skins, bromelain extract, rosemary leaf powder and ginger. They were selected based on their postulated safety and effects on various anti-inflammatory pathways in other species (Tsai *et al.*, 1999; Secor *et al.*, 2005; Aktan *et al.*, 2006; Altinier *et al.*, 2007; Gayathri *et al.*, 2007). Curcumin was also an ingredient due to its safety and anti-inflammatory activity as reported in chapters 2 and 3 in this thesis and as found in the literature (Chainani-Wu, 2003).

This study used a formulated cocktail of plant extracts in an *in vitro* chondrocyte model and in an explant model of OA. The objective of this study was to assess

cytotoxicity of the cocktail to the target cells (the chondrocytes) and determine whether non-cytotoxic concentrations could effectively reduce IL-1β-stimulated GAG and PGE_2 release from cartilage explants *in vitro*. The hypothesis of the study was that the cocktail of plant extracts would reduce IL-1β-stimulated GAG and PGE_2 release at non-cytotoxic concentrations.

Two other supplements that were intended for use in the PT field trial were also tested in the model. These were a commercially available joint health product (CP) and a placebo (PL). The CP contained forms of chondroitin sulphate and glucosamine, components which are reported to have anti-inflammatory (Chan *et al.*, 2005b) and anti-catabolic effects (Dechant *et al.*, 2005) on cartilage *in vitro*, as well as anabolic activity through stimulating proteoglycan production in chondrocytes (Bassleer *et al.*, 1998a; Bassleer *et al.*, 1998b). The PL contained alfalfa meal, a typical constituent of the domestic equine diet, and was not expected to have any significant effects on GAG release in the model. Given that the PL was a recognised feed ingredient being fed at very low levels, it was not tested for cytotoxicity. PT underwent all cytotoxicity, GAG and PGE_2 assays. However, the manufacturer of the PGE_2 assay then changed the kit and it no longer worked with the samples. Financial reasons prevented optimisation of the new assay. Consequently CP and PL were not tested for PGE_2.

4.2. Materials and Methods

Materials

Materials included a formulated cocktail of plant extracts (PT). The cocktail was composed of 30% *Boswellia serrata* extract, 18% dehydrated grape skin residue,

10% curcumin from *Curcuma longa*, 10% bromelain extract from *Ananas comosus L,* 10% rosemary leaf powder from *Rosemarinus officinalis,* 10% dried olive leaf, 5% ginger from *Zingiber officinale*, 3% vermiculite and 2% Hemp oil. All ingredients were purchased from Park Tonks Ltd and combined at MARS Horsecare UK Ltd.

A market leading, commercial joint health product (CP), known as Cortaflex (Equine America), was also evaluated. According to the product literature, the CP contained dehydrated alfalfa meal, ground rice hulls, isolated soy protein, dextrose, glycine, glutamine, chondroitin sulphate, glucuronic acid, proline, glutamic acid, sodium hyaluronate, aspartic acid, arginine, histidine, alanine, serine, valine, isoleucine, manganese sulphate, pyridoxine HCl (vitamin B6), ascorbic acid (vitamin C), sulphur, copper sulphate 5 mg/kg (added), vegetable oil, and is preserved with ammonium propionate.

Thirdly, a placebo (PL) of alfalfa meal was evaluated in the model. It was selected for being similar in appearance to that of the treatments and for being a staple horse feed constituent.

Assessing Solubility of Materials to Test in the Models

All materials were provided as they would be fed to horses, in a coarsely powdered supplement. For *in vitro* evaluation they needed to be solubilised and filter sterilised. However, due to the varied and sometimes fibrous nature of the supplements, complete solubilisation was not possible. Therefore, only the soluble component of the products (when dissolved in two different solvents; an aqueous solvent, DMEM, and an organic solvent, DMSO) were evaluated in this study.

Materials were added to DMEM at a concentration of 10mg/ml, and to DMSO at a concentration of 100mg/ml (to allow for dilution in DMEM down to the working concentrations, to reduce the amount of DMSO present in the wells). All solutions were put on a roller for 1 hour to mix them. All solutions were then sonicated on ice, using a stepped microtip with an amplitude of 40%. DMEM solutions were sonicated for 30 minutes with a 15 second pulse and 15 second interval. DMSO solutions were sonicated for less time to prevent overheating, 10 minutes with a 15 second pulse and 15 second interval.

All solutions were centrifuged at 3,000rpm at 4°C for 20 minutes. The supernatant was removed and filtered through a 0.2μm Whatman filter into 1.5ml sterile microcentrifuge tubes, before freezing at -20°C. To enable accurate estimates for the soluble components, it was necessary to assess the weight lost in the undissolved pellet from the total. Therefore, the remaining undissolved pellet was dried for 30 minutes in the oven at 160°C. The dried pellet was then weighed and the amount of substance in dissolution was calculated. Concentrations for the *in vitro* work were based on degree of dissolution in the selected solvents.

Chondrocyte Collection for the Cytotoxcity Studies

Cartilage was obtained from the MCP joints of two horses (a three-year-old cob gelding and a three-month-old welsh mountain pony filly), as previously described in chapter 3. Cartilage was washed, digested with collagenase and filtered before first expansion in culture medium (DMEM, 2% penicillin/streptomycin and 10% FBS). Confluent cells were passaged and seeded at 50,000 cells per well in 12-well plates. Only first and second passage cells were used for these studies.

Pilot Study Design- PT Cytotoxicity Assay

A pilot study on chondrocytes growing in six 12-well plates was conducted to determine approximate working concentrations for the PT. Frozen stock solutions of PT were dissolved and diluted in DMEM to the final working concentrations. However, the pilot studies were conducted before estimates of the soluble components of the stock solutions were calculated. Therefore, the pilot study used PT concentrations of 10ng/ml, 1μg/ml, 100μg/ml and 1mg/ml from the assumption that 100% of the PT was dissolved in the stock solutions. This meant that, when calculated accurately, the concentrations used differed between solvent and treatment. Thus PT was tested in DMEM at concentrations of 2.84ng/ml, 284ng/ml, 28.4μg/ml and 284μg/ml, and in DMSO at 6.69ng/ml, 669ng/ml, 66.9μg/ml and 669μg/ml. Plates were incubated at 37°C and 5% CO_2 for 24 hours before undergoing the live/dead cytotoxicity assay as described in chapter 3.

Study Design - Determining Cytotoxicity of PT in DMEM, in DMSO, and in DMEM with added DMSO

The pilot PT study showed that toxicity differed between solvents. This may have been due to something dissolving in DMSO that did not dissolve in DMEM. Alternatively, the substance could have been present in DMEM and DMSO, but the presence of DMSO permeabilised the cell membranes to the dissolved substance. Therefore, a study was designed to test the hypothesis that DMSO permeabilised the cell to the substance that was solubilised in both DMEM and DMSO. The study tested the three concentrations of PT in DMSO, PT in DMEM, and PT in DMEM with exogenous DMSO added at the same concentrations that would be present in the

PT in DMSO treated wells. Three accurately calculated PT concentrations in both DMEM and DMSO were used; 10μg/ml (not toxic in either solvent), 65μg/ml (toxicity seen in DMSO but not DMEM) and 150μg/ml (strong cytotoxicity seen in DMSO but not in DMEM). Plates were incubated for 24 hours at 37°C and 5% CO_2 before and undergoing the cytotoxicity assay as described in chapter 3.

Final Study Design - PT and CP 5 Day Cytotoxicity Test

The final cytotoxicty test using accurate concentrations used first passage chondrocytes in 12-well plates. Control wells contained culture medium alone which formed the base for the treatments. DMSO in culture medium was included as a solvent control. The NO generator, sodium nitroprusside (SNP), was dissolved in DMEM (50mM) and used as a positive control for cell death. Stock solutions (1mg/ml) of PT and CP were prepared in DMEM and DMSO and diluted in DMEM to their final working concentrations. PT test concentrations in DMSO (0.5μg/ml, 10μg/ml, 20μg/ml, 45μg/ml, 65μg/ml) and in DMEM (10μg/ml, 65μg/ml, 150μg/ml, 300μg/ml, 600μg/ml) were prepared and added to the appropriate wells. The upper limits tested for each solvent varied based on the values at which toxicity occurred after 24 hours in the pilot study (66.9μg/ml in DMSO in the pilot study), or above the level at which toxicity did not occur (284μg/ml in DMEM in the pilot study). PT concentrations were added to the appropriate wells. CP in DMEM (450ng/ml, 45μg/ml, 450μg/ml) and DMSO (400ng/ml, 40μg/ml, 400μg/ml) were also prepared and added to the appropriate wells. Plates were incubated at 37°C and 5% CO_2 for five days, with a medium change mid-way through the experiment. The removed media were spun down and the resulting pellet of cells resuspended in fresh medium containing the treatments before adding back to the appropriate well. Four plates

were assessed for toxicity at 5 days with the live/dead cytotoxicity assay as described in chapter 3.

Cartilage Collection for the Explant Studies

Macroscopically normal articular cartilage samples (selected as previously described) were obtained from the MCP joints of fifteen Thoroughbred-type horses aged between three and 12 years old. Cartilage explants were collected as described previously and equilibrated overnight (37°C/ 5% CO_2) before media were replaced with fresh media containing the treatments. All treatments were repeated in triplicate per horse and three horses were used per study.

Study Design – Effect of PT on GAG and PGE_2 Release

DMEM with 2% penicillin/streptomycin formed controls and the base medium for all treatments. DMSO controls were previously shown not to affect GAG release (see chapter 2), and were not included on the plate due to space restrictions. Recombinant equine IL-1β (10ng/ml) was added to all treatment wells.

To determine the appropriate solvent in which the PT effectively reduced GAG release, the PT treatments were evaluated in two studies, with the first study dictating the solvent and concentrations to be used in the second study. The first study tested PT in DMEM or PT in DMSO at 0.4μg/ml, 40μg/ml and 400μg/ml in 1ml medium per well containing IL-1β (10ng/ml). The volume of DMSO did not exceed 1% of the 1ml per well in the highest concentration tested. Explants were incubated for 5 days (37°C/ 5% CO_2), after which time both explants and supernatants were removed and frozen at -20°C until ready to assay. A GAG assay and a high sensitivity PGE_2

assay respectively were performed as previously described in chapter 2 (pages 53 and 93 respectively).

The second study used fresh cartilage explants to examine a narrower dose range, from physiologically relevant concentrations to those at which initial signs of adverse effects on cell viability were observed. The lowest concentration selected was lower than the published blood plasma levels after oral administration of one of the PT ingredients, curcumin, to mice (0.22μg/ml maximum plasma concentration) (Pan *et al.*, 1999). Consequently, PT in DMSO was diluted to 25ng/ml, 0.5μg/ml, 10μg/ml, 20μg/ml and 45μg/ml and added to the treatment wells to a final volume of 1ml. In this study, the maximum concentration of DMSO in the PT treated wells did not exceed 0.0005% of the 1ml per well. Positive controls were included in the second study and contained the NSAID, carprofen (Rimadyl®, Pfizer; 100μg/ml), to reduce IL-1β-stimulated GAG release. As before, explants were incubated for five days (37°C/ 5% CO_2), before both explants and supernatants were removed and frozen at -20°C until ready for GAG assay assessment.

Study Design – Effect of the Commercial Product on GAG Release

Similarly, the CP was evaluated in two studies, but only efficacy in terms of GAG release was measured due to financial reasons. The CP was first tested in DMEM and DMSO at 0.4μg/ml, 40μg/ml and 400μg/ml in 1ml medium per well containing IL-1β (10ng/ml) to determine the appropriate solvent in which the treatment effectively reduced GAG release over five days. These results led to the second study examining CP in DMEM at 25ng/ml, 0.5μg/ml, 10μg/ml, 20μg/ml and

45μg/ml on fresh cartilage explants. Positive controls were included in the second study as before.

Study Design – Effect of the Placebo on GAG Release

PL treatment was tested in DMEM and DMSO at 0.4μg/ml, 40μg/ml and 400μg/ml in 1ml medium per well containing IL-1β (10ng/ml) to determine whether the treatment in either solvent effectively reduced GAG release. After five day incubation (37°C/ 5% CO_2), the samples underwent a GAG assay as previously described (page 53).

All test substances (PT, CP and PL) were tested alone in the GAG assay for their native GAG content, which may have interfered with the assay. However, at the concentrations used for the studies in the GAG assay, the levels of GAG were low and read either near to or off the bottom of the curve (i.e. 10μg/ml), suggesting that native GAG in the samples wasn't affecting the GAG assay (appendix 9). The PT at the highest concentration tested (400μg/ml) contained 11μg GAG according to the assay but this was due to the colour of the extract affecting the result, not the presence of sulphated GAGs. Consequently, plates for the GAG assay containing PT in DMEM 400μg/ml contained a blank to account for this effect.

Statistical Analysis

For the cytotoxcity studies, six different fields of view of live and dead cells were taken per well (magnification x100), the live and dead cells were quantified with Image J Software and the percentage of dead cells calculated. Statistical analysis

was performed using a one-way ANOVA with Tukey's *post hoc* test. An adverse effect on cell viability was defined as percentage of dead cells being significantly more ($p<0.05$) than controls.

For the explant studies, percentage GAG and total PGE_2 release into the media were calculated and results were statistically analyzed using a one-way ANOVA with Tukey's multiple comparison *post hoc* test. Statistical significance was set at $p<0.05$. Values are reported as means of combined animals ± SEM.

4.3. Results

PT (≥66.9μg/ml) is Cytotoxic in DMSO After 24 Hours

The pilot study showed that the PT was cytotoxic at 66.9μg/ml when dissolved in DMSO, whereas no toxicity was seen with the PT dissolved in DMEM at concentrations up to and including 284μg/ml after 24 hours (figure 36).

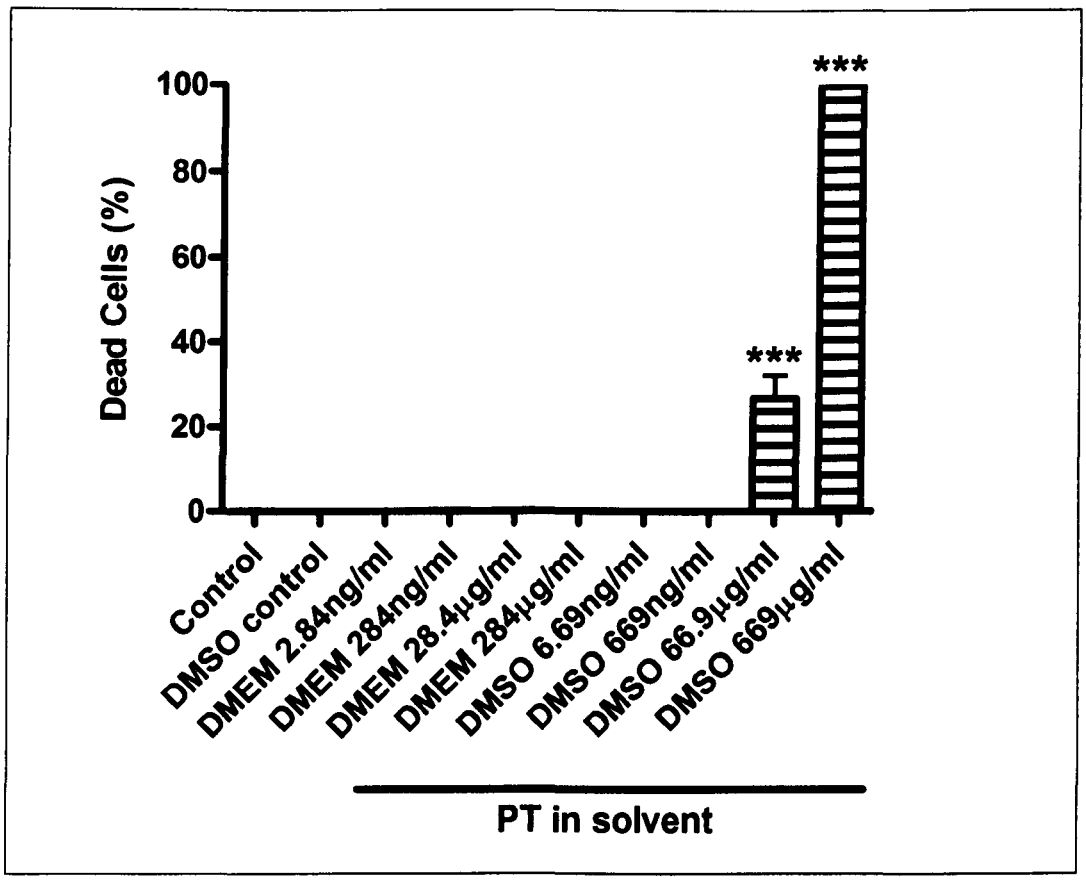

Figure 36. Pilot study chondrocyte cytotoxicity results of 24 hour culture with the plant extract cocktail (PT) solubilised in Dulbecco's modified Eagle's medium (DMEM) or dimethyl sulfoxide (DMSO) at various concentrations

*Control represents basal culture medium alone, DMSO control contains a volume of DMSO equivalent to that found in the 669µg/ml treatment. Results are expressed as the mean percentage of dead cells per field of view out of the total number of cells counted + SEM. Significance compared to control is shown by *** ($p<0.001$).*

Mean cell death in the controls after 24 hours was 0.10 ± 0.03%. The PT in DMSO significantly increased mean cell death to 26.96 ± 5.22% (66.9μg/ml) and 99.83 ± 0.05% (669μg/ml).

PT Cytotoxicity in DMSO is Caused by a Substance Not Dissolved in DMEM

The PT in DMSO at 65μg/ml caused cells to begin to round and detach, which was more pronounced at 150μg/ml (figure 37). This was not seen in the PT in DMEM alone, or with exogenous DMSO-treated cells. The cytotoxicity assay confirmed that this change in morphology seen in the PT in DMSO-treated cells at 65μg/ml and above was consistent with increased cell death (as shown in figure 38 and quantified in figure 39).

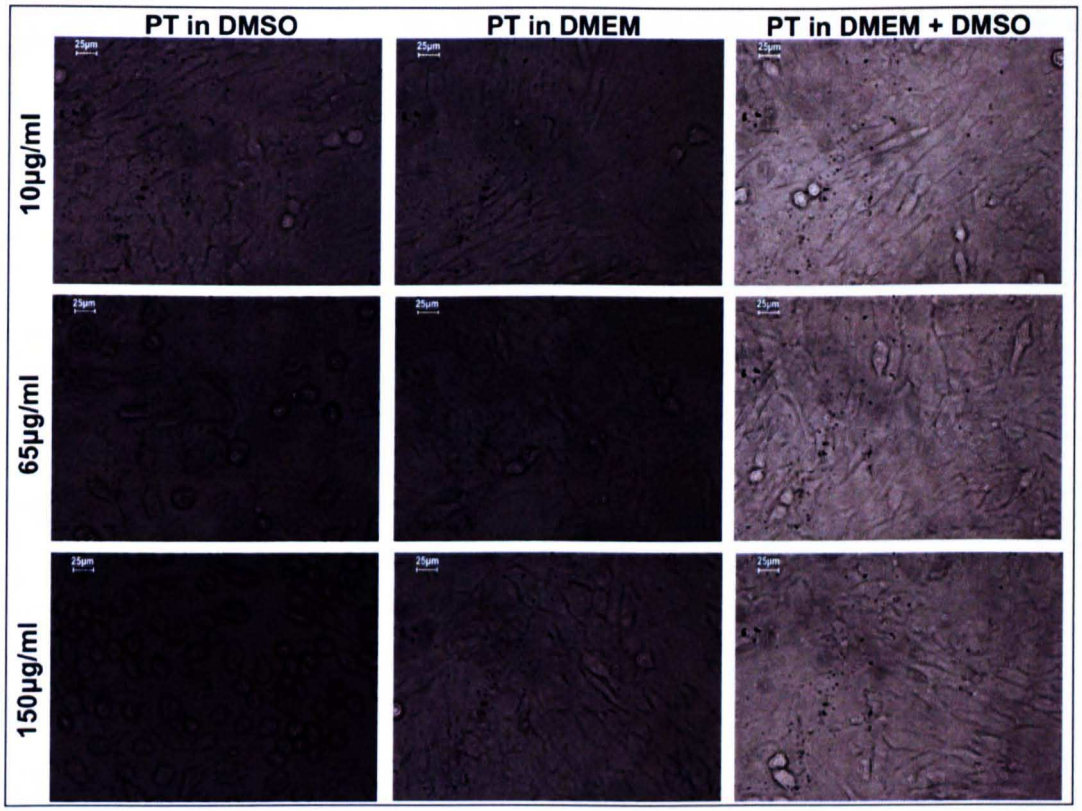

Figure 37. Morphology of primary equine chondrocytes after 24 hours with the plant extract cocktail (PT) solubilised in Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), or in DMEM with an equivalent concentration of DMSO (magnification x400)

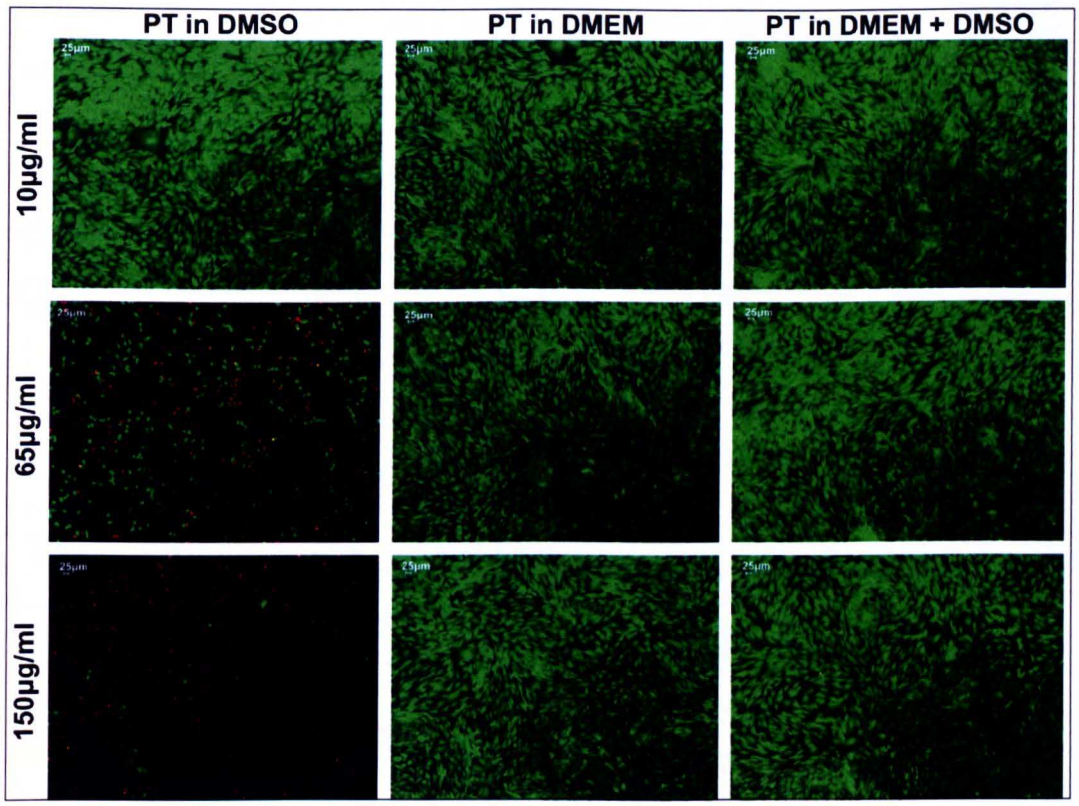

Figure 38. Viability of primary equine chondrocytes after 24 hours with the plant extract cocktail (PT) solubilised in Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), or in DMEM with an equivalent DMSO concentration (magnification x400)

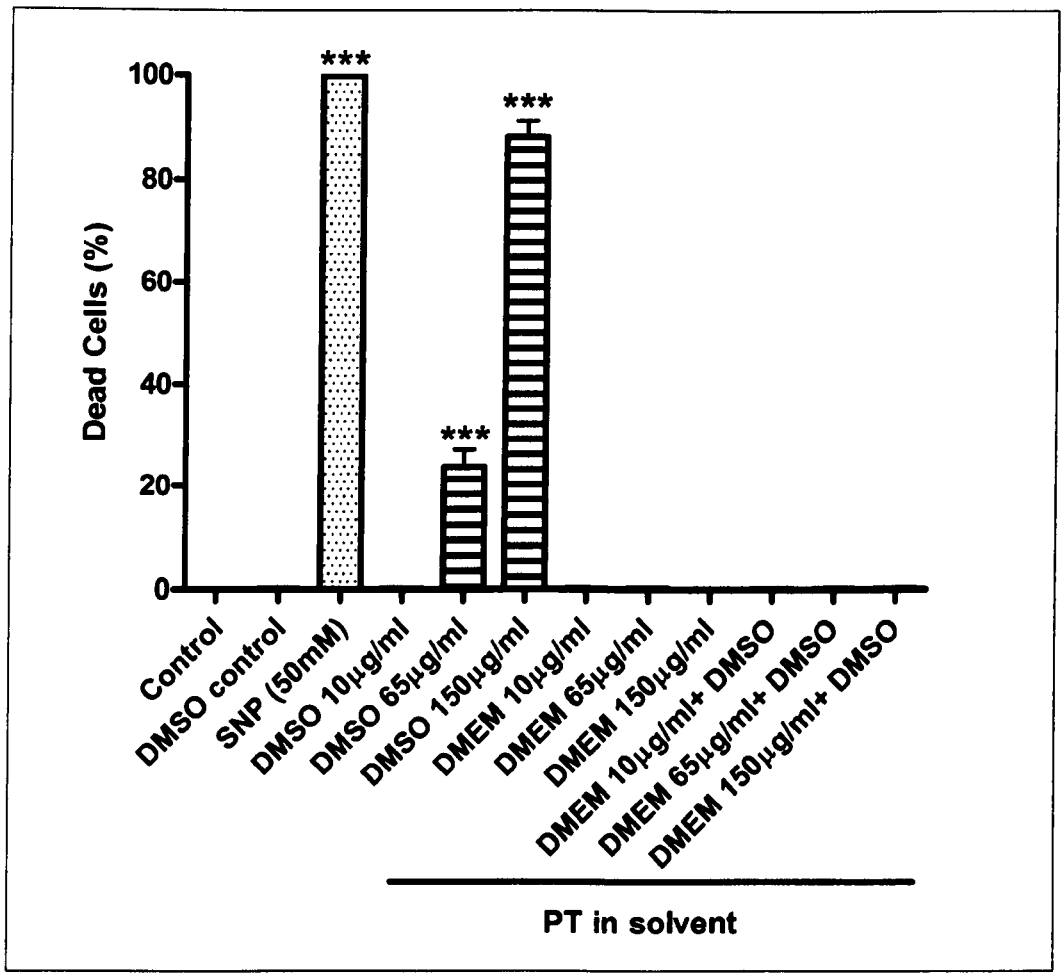

Figure 39. Percentage of dead primary equine chondrocytes after 24 hour culture with the plant extract cocktail (PT) solubilised in dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), or in DMEM with DMSO added

*Control represents basal culture medium alone, sodium nitroprusside (SNP; 50mM) was used a positive control for cell death, DMSO control contains a volume of DMSO equivalent to that found in the 150µg/ml treatment. Results are expressed as the mean percentage of dead cells per field of view out of the total number of cells counted and bars represent standard error of the means (SEM). Significance compared to control is indicated by *** ($p<0.001$).*

Control cells had a mean cell death level of 0.09 ± 0.02%, which was significantly increased by the cell-death inducing agent SNP to 99.97 ± 0.03% after 5 days. At 10μg/ml, the PT did not increase cell death compared to controls and DMSO controls when dissolved in DMSO (0.14 ± 0.03%), in DMEM (0.13 ± 0.03%) and in DMEM with exogenous DMSO (0.13 ± 0.04%). At 65μg/ml, the PT in DMSO significantly increased cell death to 23.76 ± 3.50% ($p<0.001$) whereas the PT in DMEM did not, either alone (0.23 ± 0.07%, n.s.) or in conjunction with the addition of exogenous DMSO (0.16 ± 0.09% n.s.). These results were supported by the PT at 150μg/ml which also significantly increased mean cell death in DMSO to 88.16 ± 3.07% ($p<0.001$), but did not in DMEM (0.12 ± 0.03%, n.s.) nor in DMEM with exogenous DMSO (0.19 ± 0.03%, n.s.).

PT (≥45μg/ml) is Cytotoxic in DMSO After 5 Days

Cytotoxicity of PT at five days was visualised and quantified with a cytotoxicity assay (figure 40). Control wells provided the basal level of cell death after five days (mean cell death (%) ± SEM; 0.25 ± 0.05%. SNP (50mM) significantly increased cell death to 100 ± 0.00% ($p<0.001$). DMSO did not significantly increase cell death after five days (0.59 ± 0.10%) compared to control.

PT (65μg/ml) in DMSO significantly increased cell death compared to controls (95.65 ± 1.18%, $p<0.001$) (figure 40.A). PT (45μg/ml) caused a small but significant increase 2.59 ± 1.18% mean cell death ($p<0.01$). However, PT in DMEM did not increase cell death at concentrations up to and including 600μg/ml (figure 40.B) (PT in DMEM 600μg/ml, mean cell death 0.06 ± 0.02%).

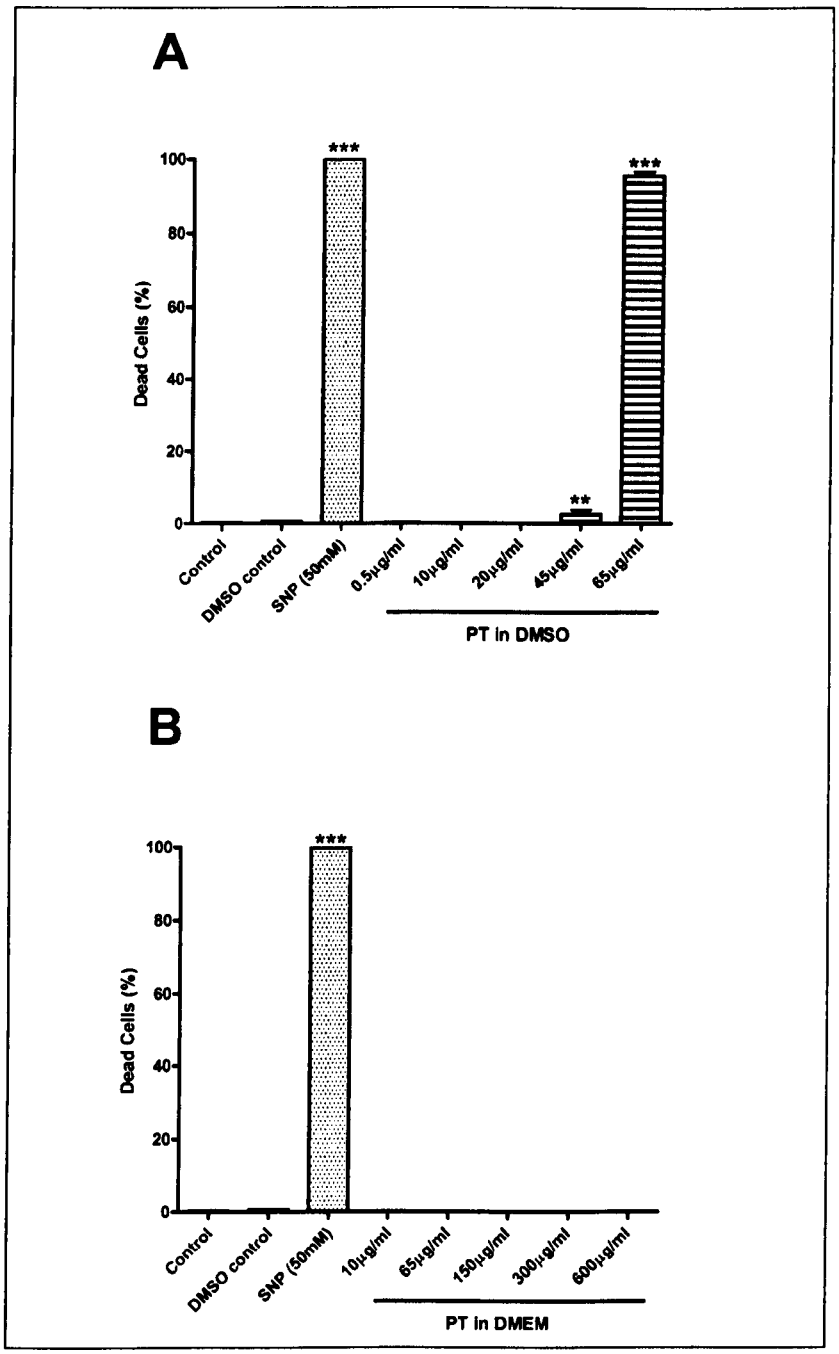

Figure 40. Mean percentage of dead primary equine chondrocytes per field of view for dose ranges of the plant extract cocktail (PT) in dimethyl sulfoxide (DMSO) (A) or Dulbecco's modified Eagle's medium (DMEM) (B) after 5 days

*Control represents basal culture medium alone, DMSO control contains a volume of DMSO equivalent to that found in the PT in DMSO 65µg/ml treatment. Results are expressed as the mean percentage of dead cells per field of view and bars represent standard error of the means (SEM). Significance compared to control is shown by ** ($p<0.01$) and *** ($p<0.001$).*

PT (≥40μg/ml) Reduces GAG Release in DMSO but Increases GAG Release in DMEM After 5 Days

Cartilage explants were stimulated with IL-1β to initiate the early stages of cartilage degradation as determined by GAG release (figure 41). Control explants released a mean GAG level of 14.07 ± 1.04% after five days in culture, which was significantly increased to 47.44 ± 5.75% with the addition of IL-1β (10ng/ml, $p<0.001$). The plant cocktail at 0.4μg/ml had no significant effect on GAG release when dissolved in DMEM at (49.38 ± 3.82%) or DMSO (40.75 ± 5.31%). In DMSO, the plant cocktail significantly decreased IL-1β-stimulated GAG release at 40μg/ml (20.91 ± 2.23%; $p<0.001$) and 400μg/ml (12.51 ± 2.26%; $p<0.001$). However, in DMEM, the plant cocktail increased IL-1β-stimulated GAG release at 40μg/ml (82.63 ± 2.42%; $p<0.001$) and 400μg/ml (90.97 ± 0.60%; $p<0.001$).

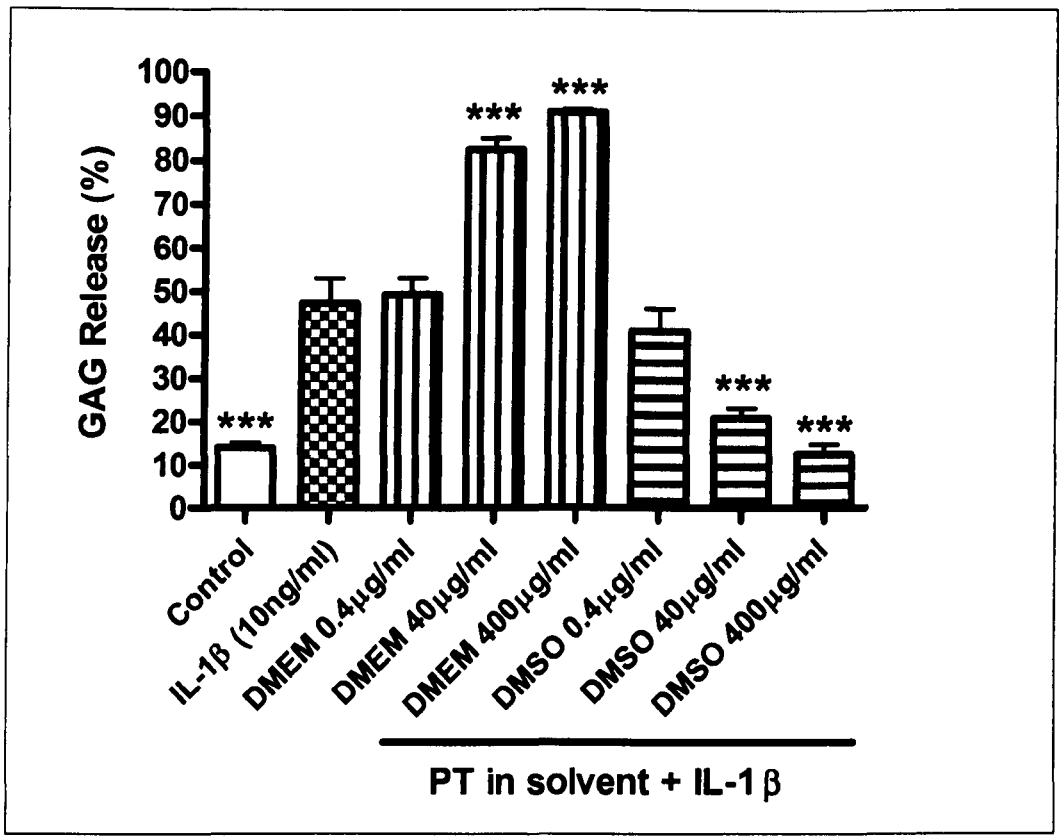

Figure 41. Percentage of glycosaminoglycan (GAG) release from interleukin-1beta (IL-1β)-stimulated equine cartilage explants after 5 day culture with the plant extract cocktail (PT) dissolved in DMEM (vertical stripes) or DMSO (horizontal stripes)

*Results are expressed as percentage GAG released and represent mean values from 3 replicates per treatment from 3 separate experiments (individual horses), i.e. n=3 per graph. Bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml; chequered column) is indicated by *** ($p<0.001$).*

PT in DMSO (≥40μg/ml) Reduces PGE_2 Release After 5 Days

The mean PGE_2 release from control explants over 5 days was 14.10 ± 1.08pg/ml. Explants released 434.20 ± 109.30pg/ml PGE_2 in response to IL-1β stimulation (figure 42). The plant cocktail in DMEM did not significantly affect IL-1β-stimulated PGE_2 release at 0.4μg/ml or 40μg/ml, however it significantly reduced PGE_2 release at 400μg/ml (78.19 ± 27.80pg/ml; $p<0.01$). In DMSO, the plant cocktail significantly reduced PGE_2 release from IL-1β-treated explants at 40ug/ml (70.83 ± 7.85pg/ml; $p<0.01$) and 400μg/ml (86.21 ± 6.65pg/ml; $p<0.01$).

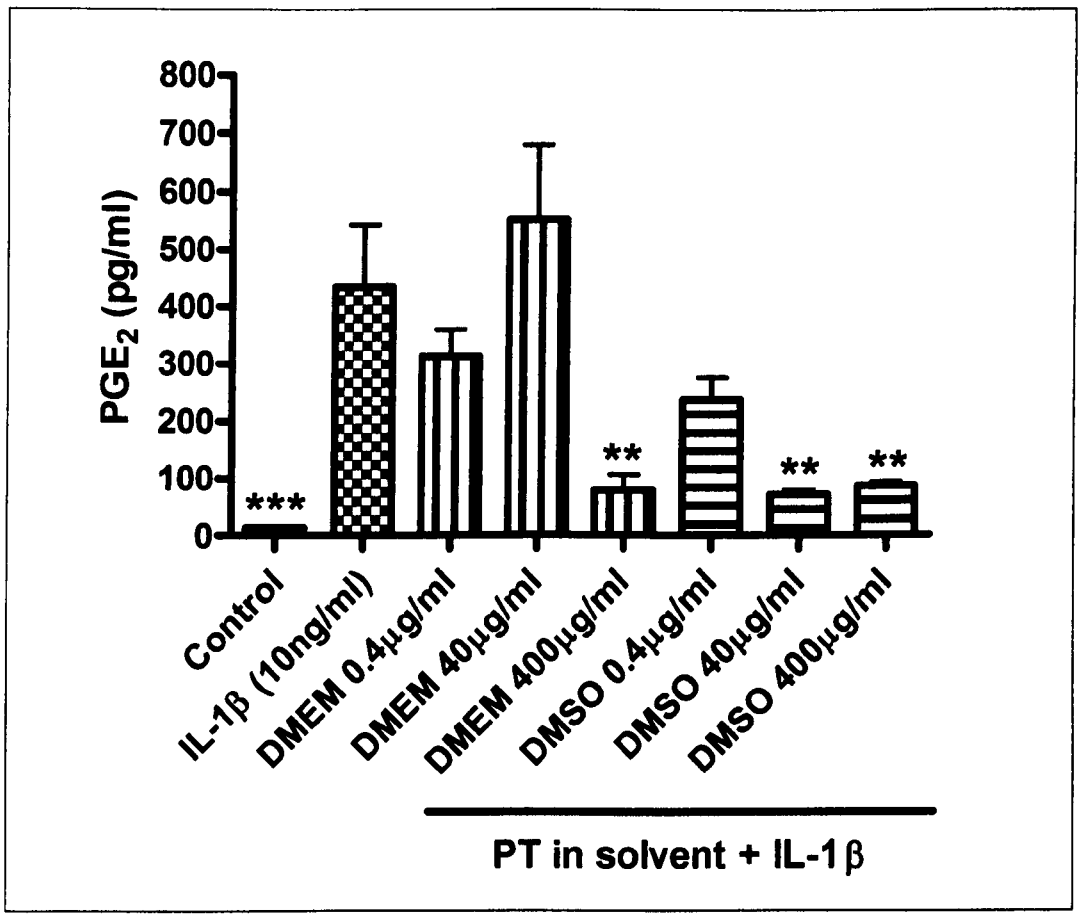

Figure 42. Prostaglandin E_2 (PGE_2) release from interleukin-1beta (IL-1β)-stimulated explants after 5 days with the cocktail of plant extracts (PT) in Dulbecco's modified Eagle's medium (DMEM) (vertical stripes) or dimethyl sulfoxide (DMSO) (horizontal stripes)

*Control indicates explants incubated in culture medium alone. Values are reported as the mean of 3 horses per treatment and bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml; chequered column) is indicated by ** ($p<0.01$) and *** ($p<0.001$).*

Non-Cytotoxic PT Concentrations (10μg/ml and 20μg/ml) in DMSO Reduce GAG Release

From these initial results, the PT underwent a further study in DMSO as this was the solvent which effectively reduced IL-1β-stimulated GAG release. This was done, to determine the effect of PT at concentrations ranging from physiologically achievable to the lowest concentration at which cytotoxicity occurred in either solvent (i.e. 25ng/ml to 45μg/ml).

IL-1β significantly increased GAG release compared to control ($p<0.001$) (figure 43). This release was significantly reduced by the NSAID ($p<0.01$). PT in DMSO significantly reduced GAG release from IL-1β stimulated explants at 10μg/ml ($p<0.05$), 20μg/ml and 45μg/ml (both $p<0.001$)

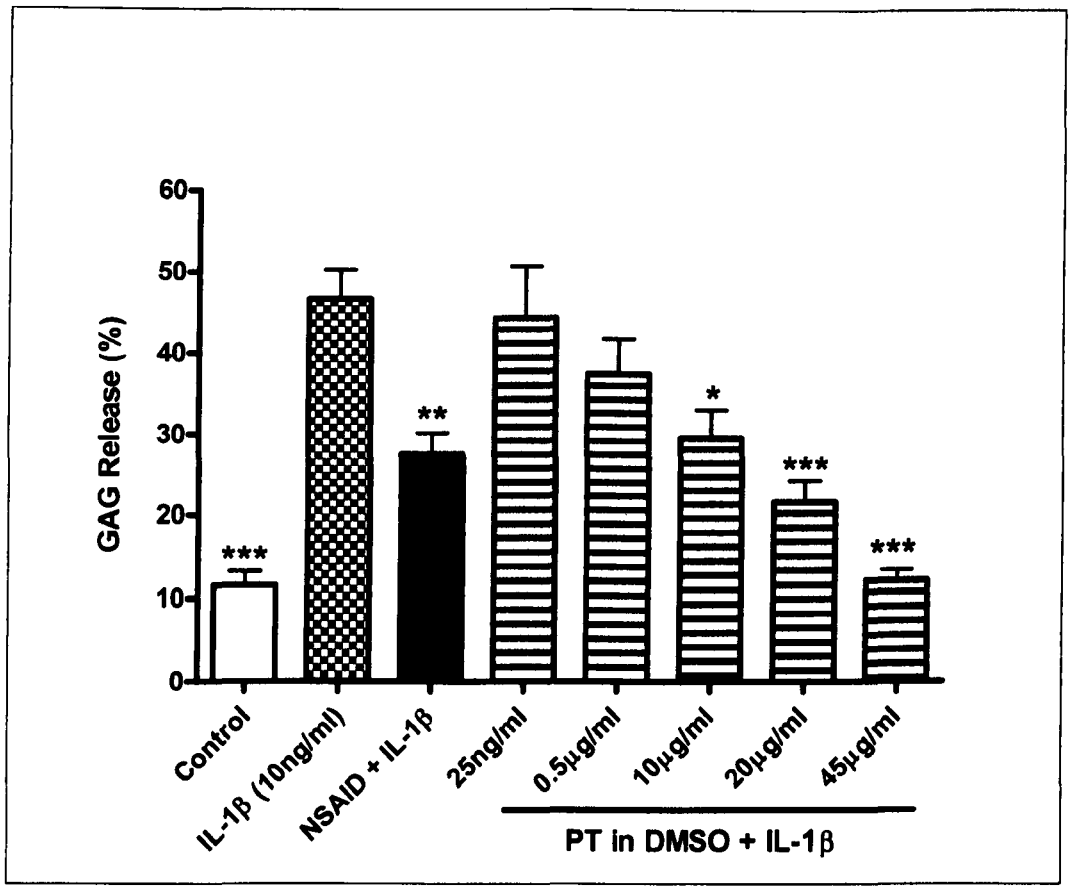

Figure 43. Percentage of glycosaminoglycan (GAG) release from cartilage explants in response to interleukin-1beta (IL-1β; 10ng/ml) and a nonsteroidal anti-inflammatory drug (NSAID; 100µg/ml) or the plant extract cocktail (PT) dissolved in DMSO

*Results are expressed as percentage GAG released and represent mean values from 3 replicates per treatment from 3 separate experiments (individual horses). Bars represent standard error of the means (SEM). Significance compared to IL-1β (chequered column) is indicated by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).*

CP (450μg/ml) is Cytotoxic in DMEM After 5 Days

The CP was tested alongside PT on the same plates therefore the control, DMSO control and SNP mean cell death values in this section are the same as previously stated for the PT cytotoxicity results, i.e. 0.25 ± 0.05% (control); 0.59 ± 0.10% (DMSO control); and 100 ± 0.00% (SNP).

CP in DMSO did not increase cell death at concentrations up to and including 400μg/ml (CP in DMSO 400μg/ml, mean cell death 0.28 ± 0.08%) (figure 44.A). However, CP (450μg/ml) in DMEM caused a small but significant increase in cell death after five days to 4.6 ± 1.03% ($p<0.001$) compared to control (figure 44.B).

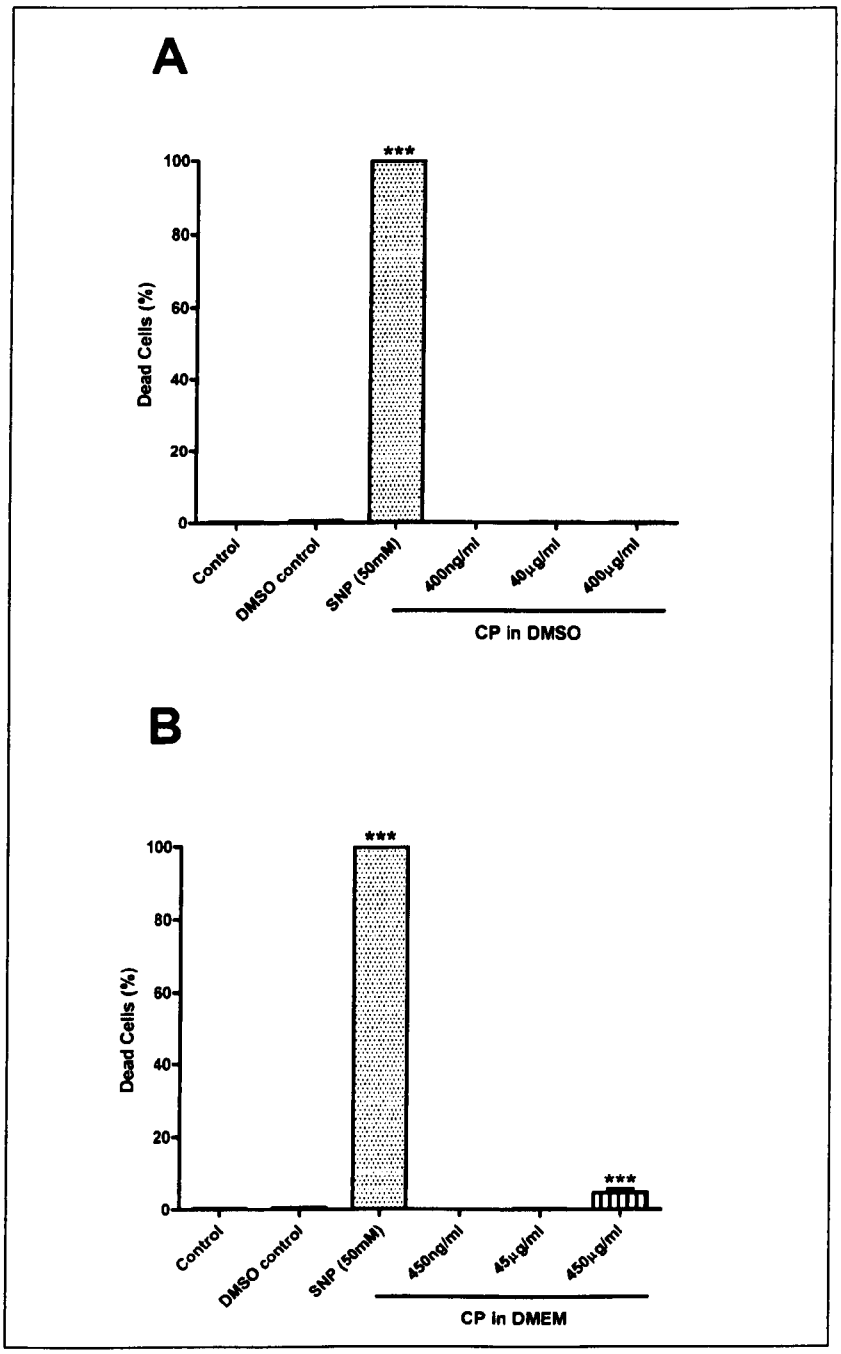

Figure 44. Graphs showing the mean percentage of dead cells per field of view for the commercial product (CP) in dimethyl sulfoxide (DMSO) (A) or Dulbecco's modified Eagle's medium (DMEM) (B) after 5 days

*Control represents basal culture medium alone, DMSO control contains a volume of DMSO equivalent to that found in the CP in DMSO 400µg/ml treatment. Results are expressed as the mean percentage of dead cells per field of view and bars represent standard error of the means (SEM). Significance compared to control is shown by *** ($p<0.001$).*

CP in DMEM (≥40μg/ml) Significantly Reduces GAG Release

The CP in DMSO had no significant effect on GAG release, but CP dissolved in DMEM significantly reduced IL-1β-stimulated GAG release at 40μg/ml ($p<0.001$) and 400μg/ml ($p<0.01$) (figure 45).

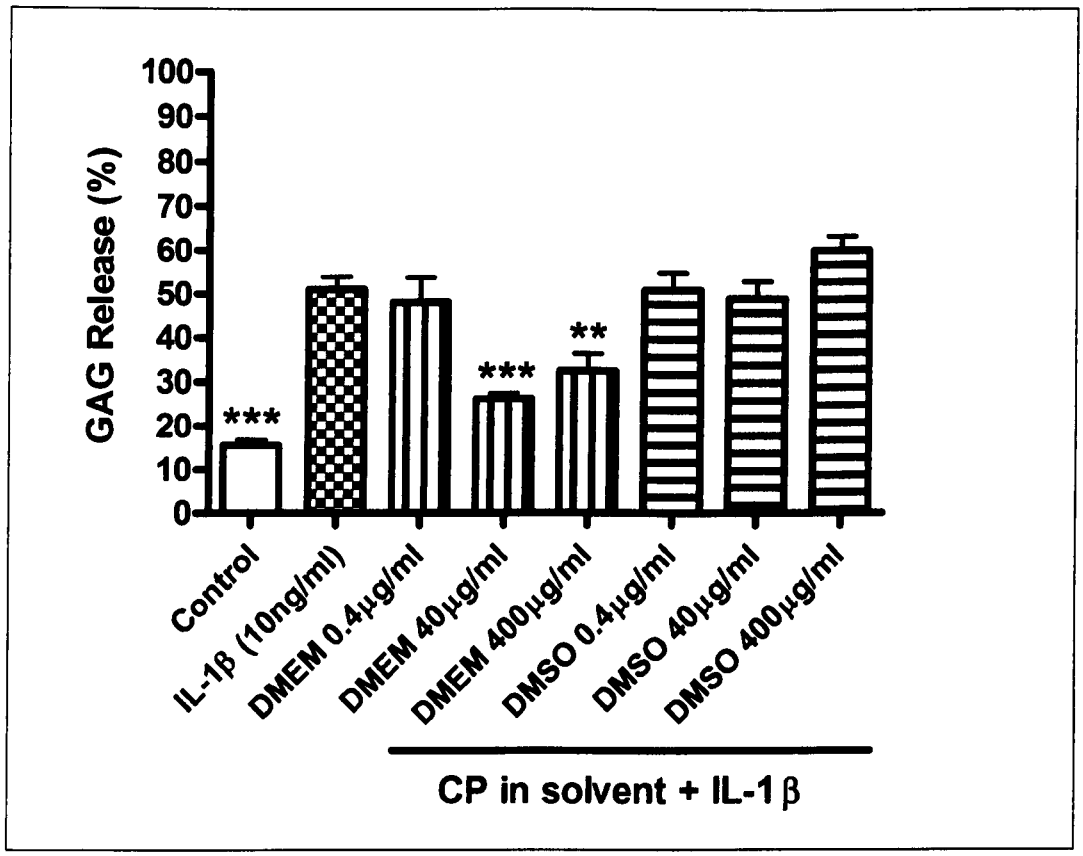

Figure 45. Percentage of glycosaminoglycan (GAG) release from interleukin-1beta (IL-1β)-treated cartilage explants after 5 days with the commercial product (CP) in Dulbecco's modified Eagle's medium (DMEM) (vertical stripes) or dimethyl sulfoxide (DMSO) (horizontal stripes)

*Results are expressed as mean percentage GAG released from 3 replicates per treatment from 3 separate experiments (individual horses), i.e. n=3. Bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml; chequered column) is indicated by ** ($p<0.01$) and *** ($p<0.001$).*

The CP then underwent a further study in DMEM, as this was the solvent which effectively reduced IL-1β-stimulated GAG release. IL-1β significantly increased GAG release compared to control ($p<0.001$) (figure 46). This release was significantly reduced by the NSAID ($p<0.01$). CP in DMEM significantly reduced GAG release at concentrations of 10μg/ml and above ($p<0.001$).

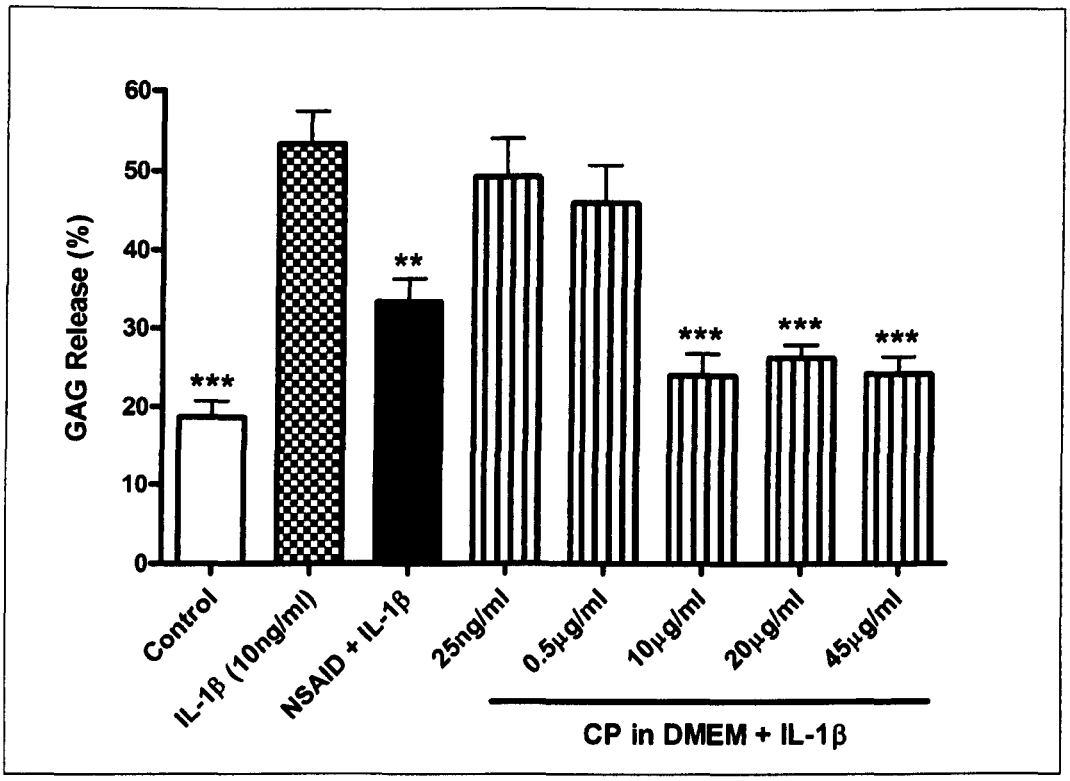

Figure 46. Percentage of glycosaminoglycan (GAG) release from cartilage explants in response to interleukin-1beta (IL-1β; 10ng/ml) and a nonsteroidal anti-inflammatory drug (NSAID; 100µg/ml) or the commercial product (CP) dissolved in Dulbecco's modified Eagle's medium (DMEM)

*Results are expressed as mean percentage GAG released from 3 replicates per treatment from 3 separate experiments (individual horses). Bars represent standard error of the means (SEM). Significance compared to IL-1β (chequered column) is indicated by ** ($p<0.01$) and *** ($p<0.001$).*

PL Does Not Significantly Affect GAG Release After 5 Days

Although there were trends for the placebo to reduce IL-1β-stimulated GAG release in DMEM and increase GAG release in DMSO, no results were significant. Therefore, PL did not significantly reduce IL-1β-stimulated GAG release in either DMEM or DMSO at concentrations up to 400μg/ml (figure 47).

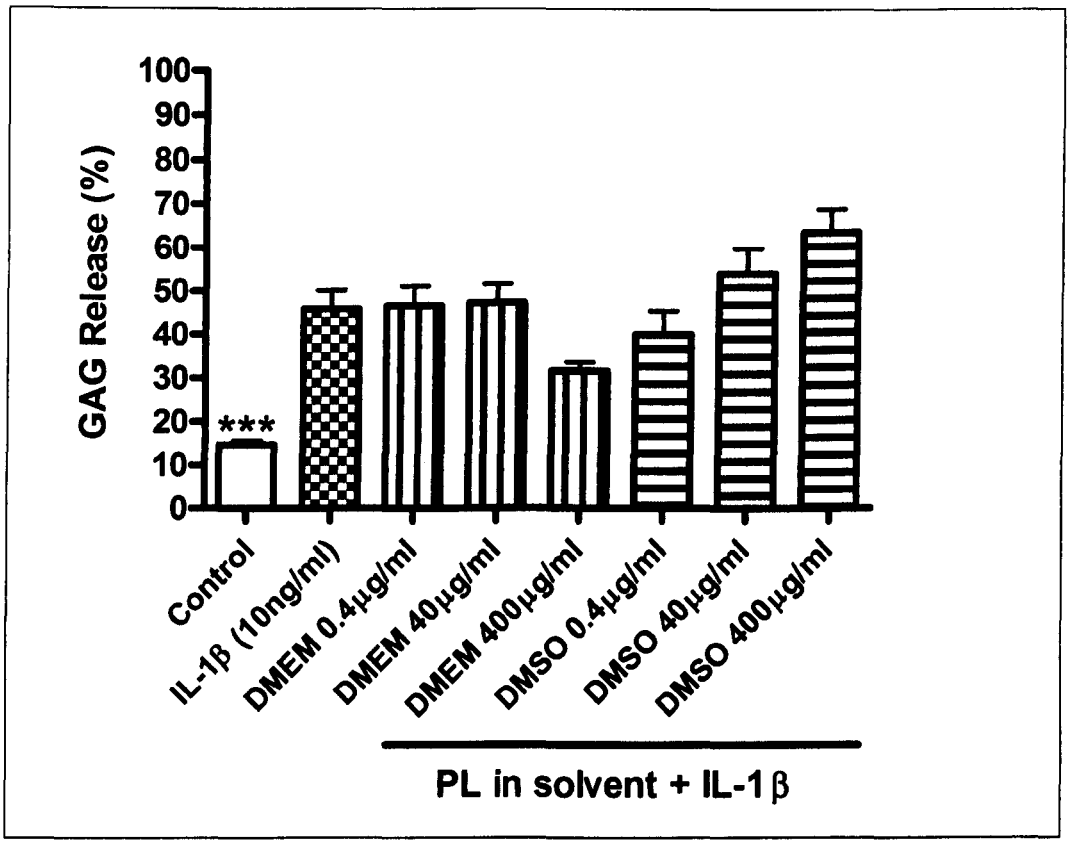

Figure 47. Percentage of glycosaminoglycan (GAG) release from interleukin-1beta (IL-1β)-stimulated cartilage explants after 5 days with the placebo (PL) dissolved in Dulbecco's modified Eagle's medium (DMEM) (vertical stripes) or dimethyl sulfoxide (DMSO) (horizontal stripes)

*Results are expressed as mean percentage GAG released from 3 replicates per treatment from 3 separate experiments (individual horses), i.e. n=3. Bars represent standard error of the means (SEM). Significance compared to IL-1β (10ng/ml; chequered column) is indicated by *** ($p<0.001$).*

4.4. Discussion

The objectives of the work described in this chapter were to test the PT for cytotoxicity and efficacy in terms of reducing IL-1β-stimulated GAG and PGE_2 release in an *in vitro* explant model of early OA. Initial 24 hour pilot studies with the PT found that it was cytotoxic to monolayer equine chondrocytes when dissolved in DMSO at 66.9μg and above, whereas in DMEM, concentrations up to 284μg/ml showed no adverse effects on chondrocyte viability. This may have been due to an ingredient solubilised in DMSO that was not soluble in DMEM. However, it is well known that DMSO permeabilises cell membranes through pore-formation (Notman *et al.*, 2006; Gurtovenko and Anwar, 2007). Thus, although the material may have been solubilised in DMEM, the absence of DMSO may have prevented it entering the cell, thereby accounting for the differing toxicities between solvents. In order to test this, non-toxic and toxic concentrations of PT in DMSO and DMEM were tested along with PT in DMEM with an equivalent concentration of added DMSO to that found in the PT in DMSO treated wells. The results showed that concentrations of PT that were toxic in DMSO, were not toxic when dissolved in DMEM both with and without the presence of DMSO. Therefore, it was considered that the toxicity seen in the PT in DMSO-treated cells was more likely a solubility issue than due to the permeabilising activity of DMSO.

Further tests with PT over five days showed that in DMSO initial signs of cytotoxicity were present at concentrations of 45μg/ml, whereas in DMEM concentrations up to 600μg/ml showed no signs of toxicity. In terms of efficacy, the PT was able to reduce IL-1β-stimulated GAG release, at concentrations non-detrimental to chondrocyte viability. However, as with the cytotoxicity testing, this was affected by the choice of solvent used. The PT showed positive anti-catabolic

effects when in DMSO. DMSO controls confirmed that this was not due to the effect of the DMSO, which has known anti-inflammatory effects. However, some of the actives in the ingredients of the PT, such as resveratrol in grape skins and curcumin in turmeric, are known to be soluble in organic solvents such as DMSO and show little, if any, solubility in aqueous solutions (Iwuchukwu and Nagar, 2008; Henrotin *et al.*, 2010). Thus, the beneficial effects of the DMSO soluble-actives would not be present in the DMEM preparation.

Similarly, the effect of solvent on supplement effects was observed when increased GAG (82% of the total GAG present at concentrations of 40μg/ml PT) was released from explants treated with PT in DMEM but not DMSO. This could be attributed to the presence of bromelain, a mixture of water-soluble, stable, proteolytic enzymes extracted from the pineapple plant (Hale *et al.*, 2005), which are likely to be active and able to break down the cartilage matrix, under culture conditions of 37°C. Consequently, when developing models to test supplements, their modes of action and solubility in different solvents must be considered when interpreting the results. Testing a cocktail of plant extracts with different actions in the model highlighted these issues sooner than testing individual ingredients would have done.

Although this study was unable to show beneficial effects of PT in DMEM, in terms of reduced GAG release, it did significantly reduce IL-1β-stimulated PGE_2 release at 400μg/ml. This agrees with other studies reporting the anti-inflammatory ability of bromelain *in vivo* (Secor *et al.*, 2005). However, this anti-inflammatory effect is accompanied with significant GAG loss in the equine explant model, which is indicative of cartilage breakdown. This suggests either that the cocktail in DMEM is not supportive to joint health, or that the model and the GAG assay were unsuitable for evaluating certain components of the cocktail, such as bromelain.

The CP showed toxicity in DMEM at 450μg/ml but not in DMSO at concentrations up to 400μg/ml, suggesting that the water-soluble components in the CP were cytotoxic compared to the soluble components in the organic solvent. In DMEM, the CP significantly reduced IL-1β-stimulated GAG release from cartilage explants at 40μg/ml whereas equivalent concentrations of CP in DMSO had no effect. Further studies confirmed that this effect was seen at concentrations as low as 10μg/ml. The CP is marketed as a glucosamine and chondroitin sulphate-based product. Glucosamine hydrochloride has been investigated for its anti-catabolic effects *in vitro*, and has been shown to reduce recombinant equine IL-1β–stimulated GAG release from equine cartilage explants at 25mg/ml (Fenton *et al.*, 2002). Similarly, glucosamine hydrochloride in combination with chondroitin sulphate at 250μg/ml each, significantly reduced GAG degradation in equine explants stimulated with recombinant human IL-1α (40ng/ml) (Dechant *et al.*, 2005). The proportions of glucosamine and chondroitin sulphate in the CP are unknown, although the lack of native GAG at the highest CP concentration tested, suggests that levels of chondroitin sulphate are low in the preparations used. Despite this, the CP as a whole product showed a significant reduction in IL-1β-stimulated GAG loss at 40μg/ml, lower than the concentrations used in the aforementioned studies. Thus, suggesting that the CP has anti-catabolic properties, which may be due to the forms of glucosamine and chondroitin sulphate that it contains. It should be noted here, that the reduction in GAG loss shown in this study may reflect reduced proteoglycan synthesis by the matrix, which may not be beneficial. Further studies on the effects of the CP and PT on GAG synthesis are required to answer this.

The placebo did not show any beneficial effect in terms of GAG release, suggesting that it was suitable for use as a placebo.

In conclusion, the model was able to detect a beneficial effect of PT and CP in terms of reducing IL-1β-stimulated cartilage GAG release. Although the degree of solubilisation and the solvent used affected this activity. Measuring GAG and PGE_2 alone provide a limited insight into what is happening in the model and does not address the complexities seen when working with the combination product. Thus, the ability to identify multiple cartilage protein products released from the explants in this system may increase the understanding of the processes occurring in this early OA model, and may enable a more comprehensive assessment of the effects of plant extracts tested in it.

CHAPTER 5. ADAPTING THE MODEL FOR PROTEOMIC ANALYSIS OF THE CARTILAGE SECRETOME

5.1. Introduction

The previous chapter highlighted that measuring more parameters *in vitro* may provide further information on the effects of plant extracts in different solvents in the model. One approach to achieve this is to characterise the proteins released from the cartilage (the secretome) in the early OA explant model. The value of proteomics is that proteins can be identified using database analysis and potentially quantified allowing an insight into what processes are involved, and to discover which processes may be modulated with plant extracts.

OA involves the loss of structural constituents from the articular cartilage ECM via increased cartilage catabolism and consequent failure of the chondrocytes to maintain tissue integrity (Aigner *et al.*, 2007). The degradation and release of proteins and glycoproteins may aid the diagnosis of OA. For example, increased levels of COMP and levels of COMP fragments in the synovial fluid can distinguish OA horses from normal horses (Arai *et al.*, 2005). Similarly, increased urinary COMP has identified horses with aseptic joint disease compared to animals free from joint disease (Misumi *et al.*, 2006). The levels of COMP released from OA cartilage can also vary according to the severity of the disease process. For example, elevated serum COMP is correlated with OA severity in humans (Clark *et al.*, 1999). Consequently, the ability to detect biomarkers of cartilage degradation and/ or inflammation in biological samples, such as serum, urine or synovial fluid, may enable clinicians to diagnose sub-clinical OA, determine disease stage and assess

response to therapy in human patients and companion animals. The complexity of OA means that combinations of biomarkers may be more effective in achieving these goals than single ones (Williams, 2009). Identifying these biomarkers will also aid drug discovery and drug safety/ efficacy monitoring in patients and in animal models.

Proteomics is being increasingly applied in basic cartilage biology and OA research. Characterisation of cell lysates from isolated chondrocytes has yielded valuable information regarding the soluble protein fraction of the chondrocyte proteome, and paved the way for future studies on cartilage pathologies such as OA (Ruiz-Romero *et al.*, 2005). Recently, proteomics has been used to identify differences in the intracellular proteome of chondrocytes in response to IL-1β or TNF-α (Cillero-Pastor *et al.*, 2010), and the effects of glucosamine and chondroitin sulphate on intracellular mechanisms in IL-1β-stimulated chondrocytes (Calamia *et al.*, 2010). Studies of soluble proteins in cartilage tissue from OA patients has increased the knowledge of the proteins contained within the ECM of diseased versus normal tissue (Wu *et al.*, 2007). Similarly, the synthesis and release of proteins from human OA cartilage explants have recently been examined (Polacek *et al.*, 2010). However, only a few papers have reported on proteins secreted from the cartilage ECM in response to a controlled pathological insult, such as IL-1α and all-trans-retinoic acid-treated mouse cartilage (Wilson *et al.*, 2008), and IL-1β, TNF-α and mechanical compression treated bovine explants (Stevens *et al.*, 2008). Identifying proteins released from cartilage in a serum-free model therefore has the potential to give an indication of proteins likely to be present in the synovial fluid or blood of patients in the early stages of OA, especially when the ECM has been structurally compromised or exposed to inflammatory mediators.

The present study used healthy equine cartilage explants cultured in serum-free media, either alone (representing healthy cartilage), in the presence of recombinant equine IL-1β to replicate the early inflammatory stages of OA, or a combination of IL-1β and carprofen, a NSAID, to simulate anti-inflammatory pharmacotherapy. The aim of this chapter was to use proteomic analysis of the explant culture media to identify the proteins secreted from IL-1β treated and untreated equine explants in the model, with a view to improving its applicability for evaluating the effects of plant extracts for improving joint health. The primary hypothesis of the study was that this proteomic approach could be used to detect proteins specifically secreted in response to stimulation with pathophysiologically relevant stimuli, such as IL-1β.

5.2. Material and Methods

Cartilage Explant Culture

Normal articular cartilage from the weight bearing regions of the MCP joints of three horses were used for this study. Cartilage shavings of equal thickness were aseptically harvested into low glucose (1g/L glucose) DMEM containing 4% penicillin/streptomycin before being washed twice in PBS for 20 minutes. Cartilage shavings from each animal were cut into 3mm discs using a sterile biopsy punch and five discs/ well were placed into 18 wells of a 24-well plate, containing 1ml of DMEM supplemented with 2% penicillin/streptomycin. Plates were incubated overnight (37°C/ 5% CO_2). Cell culture media were then replaced with fresh media before the experiment began.

Study Design

Explants from three individual animals were subjected to three treatments; control, IL-1β, NSAID + IL-1β (six sample replicates per treatment) totalling 18 samples per animal, equalling 54 samples in total. All wells contained 1ml of culture medium. Control wells contained the culture medium alone. Recombinant equine IL-1β (10ng/ml) was added to the remaining wells to induce cartilage inflammation. IL-1β alone formed the negative control and the addition of carprofen (100μg/ml; Rimadyl®, Pfizer), to the remaining IL-1β-treated wells acted as a positive control to counteract the IL-1β-stimulated inflammation. Explants were incubated at 37°C and 5% CO_2 for six days. After six days, the supernatants were removed and frozen at -20°C.

Trypsin Digestion of Soluble Proteins

Dithiothreitol (DTT, Sigma-Aldrich) was added to 100μl of sample to a final concentration of 10mM and incubated at 37°C for 30 minutes. Iodoacetamide (Sigma-Aldrich) (55mM) was added to each sample and incubated for 45 minutes at 37°C in the dark. Ice-cold acetone (Sigma-Aldrich) was added to each tube and mixed well. Samples were left on ice for 1 hour, vortexing for 10 seconds every 15 minutes. The precipitate was then centrifuged at 15,000 x *g* for 5 minutes at 4°C and the supernatant discarded. The protein pellet was air dried and re-suspended in 50mM ammonium bicarbonate (Sigma-Aldrich) solution to a final concentration of 10μg protein/mL. Mass spectrometry grade trypsin (Promega) was added to each tube at a ratio of 1:50 enzyme/target protein at 37°C overnight. The reaction was

stopped by adding 1µl formic acid (Sigma-Aldrich). Samples were stored at -80°C until analysis by electrospray ionisation (ESI) mass spectrometry (MS).

Analysis by Liquid Chromatography and Tandem Mass Spectrometry

Liquid chromatography (LC) was performed using an Easy-nLC (Bruker UK Ltd) under the control of Hystar (Bruker UK Ltd). Peptides generated by tryptic digestion were prepared for LC-MS/MS as follows; 2µl digest was added to 48µl solvent A (95% (v/v) H_2O, 5% (v/v) acetonitrile (Sigma-Aldrich), 0.1% (v/v) formic acid). A volume (5µl) of this dilution was then loaded onto a C18 Pepmap column (75µm ID, 15cm, LC Packings, Dionex). Peptides were separated at a flow rate of 300nl/min and the introduction of solvent B (95%(v/v) acetonitrile, 5% (v/v) water, 0.1% (v/v) formic acid) over a 30 minute linear gradient (solvent B went from 0% to 5% in 30 seconds, then from 5% to 55% in 22 minutes, up to 95% in 4 minutes, was held at 95% for 2 minutes 30 seconds to wash the column, then returned to 5% over 1 minute followed by 10 minute equilibration in 5% before the next injection, total run time 40 minutes). The LC system was interfaced directly with a 3-D high capacity ion trap (HCT) mass spectrometer (amaZon, Bruker Daltonics) via the nanoESI spray source and a target of 200,000 was set for Smart ion charge control (ICC)™.

Up to 5 precursor ions above a threshold of 10,000 were selected per MS scan. Each precursor was fragmented twice and then the mass was excluded for 1 minute. Singly charged ions were excluded. Smart parameter setting (SPS) tuning was allowed for transmission of the precursor into the trap. SmartFrag™ controlled the fragmentation of each precursor ion and this was achieved using helium gas and a 30-200% collision energy range with amplitude 1.3V.

Data Processing and Analysis

Raw LC-MS/MS data were processed automatically and Mascot compatible files (*.xml) were created using DataAnalysis^{TM} 4.0 software (Bruker UK Ltd) with the following parameters: compounds (autoMS) threshold 10,000, unlimited number of compounds, one minute retention time windows for C18 (30 min gradient).

A database search was performed using Mascot (Perkins *et al.*, 1999) software and the SwissProt database (version 5.16), with the following parameters: 2+ and 3+ ions, peptide tolerance 0.5 Da, ^{13}C=1, fragment tolerance 0.5 Da, missed cleavages: 1, instrument type: ESI-TRAP. Fixed modifications: carbamidomethyl cysteine and variable modifications: oxidation of methionine, deamidation of asparagine and glutamine residues.

Data from the Mascot search are expressed as a Mowse score. This is a statistically calculated weighting score for each peptide match, generated from comparing experimental data with the calculated peptide mass or fragment ion mass value for each entry in a sequence database (Pappin *et al.*, 1993). Higher Mowse scores result from increasing numbers of matched peaks and increase the chance that the identification is real and not random. Mowse scores lower than 40 were inspected manually and only included in the statistics if a series of at least 4 continuous fragment ions were observed. However, proteins with less than 4 continuous fragments ions were also recorded out of interest.

A schematic overview of the study design is shown in figure 48.

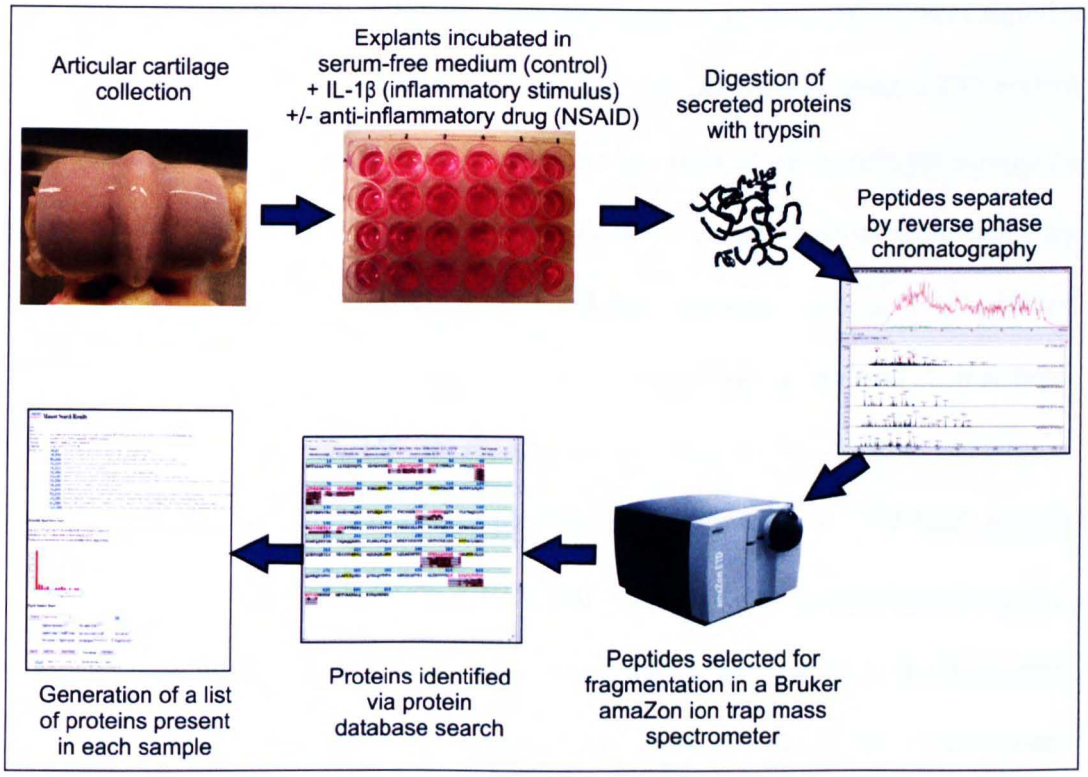

Figure 48. Schematic overview of the proteomic study design

Western Blotting

Replicate samples for each treatment were combined to give a total of three samples per horse. The protein content of each sample was quantified using a DC protein assay (as described on page 93) and concentrated to yield 50μg protein per sample by means of a freeze-dryer (Heraeus-Christ). Positive tissue controls were made by crushing equine cartilage shavings with liquid nitrogen into a fine powder. Housekeeping controls were not used as cellular housekeeping proteins such as beta-actin were inappropriate for these studies of the ECM protein secretome. Supernatant and cartilage control samples were re-suspended in 37μl LDS sample buffer (Invitrogen) and loaded onto 4-12% NuPAGE® buffered gradient pre-cast Bis-Tris gels (50μg protein/ lane. A full range molecular weight marker (GE Healthcare) was loaded (5μl) onto each gel. Samples were electrophoresed for 1 hour under denaturing and reducing conditions. Gels were either silver stained with a silver stain kit (Bio-Rad Laboratories) to observe the protein profiles of the samples, or electro-transferred onto PVDF membranes for western blotting. After blocking, primary antibodies were diluted as recommended by the manufacturer in 5% milk powder in TBS/Tween and added to the membrane for overnight incubation at 4°C. A variety of primary antibodies were selected for validating the secreted proteins discovered by the proteomic analysis.

Cartilage intermediate layer protein-1 (CILP-1) blots were performed using a sheep antibody (1:300 dilution, R&D Systems). Clusterin (1:500 dilution, Santa Cruz) and MMP-3 (1:1000 dilution, Abcam) were goat polyclonal antibodies. A rabbit polyclonal to MMP-1 was used at 1:5000 dilution (Abcam). Thrombospondin (TSP) (1:1000 dilution, Abcam) and TSP-1 (1:50 dilution, Santa Cruz) were mouse monoclonal antibodies. All antibodies were produced against human peptide

sequences with 99-100% identity to their counterparts in *Equus caballus*. The peptide sequences used by each manufacturer for antibody production were checked using the NCBI Basic Local Alignment Search Tool (Protein BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

Appropriate secondary antibodies conjugated to HRP were used; polyclonal rabbit anti-sheep HRP (diluted 1:10000, Abcam), polyclonal rabbit anti-goat Immunoglobulins/HRP (diluted 1:10000, Dako) and goat anti-mouse/anti-rabbit labelled polymer HRP (diluted 1:400, Dako).

After rinsing the membranes, chemiluminescence was detected using ECL+ on a Typhoon Trio+ Variable Mode Imager. Densitometric quantification of the Western blot bands was performed using ImageJ software. The relative intensity of protein bands in IL-1β and NSAID + IL-1β treated samples were compared to those in control samples from each horse

5.3. Results

Differing Media Protein Profiles from Control, IL-1β and NSAID-treated Samples

An initial screen was used to visually assess whether the various treated samples had different protein profiles. Silver staining of one-dimensional (1-D) gels revealed differences in the secretome protein profiles between control, IL-1β-treated and IL-1β plus NSAID-treated samples (figure 49).

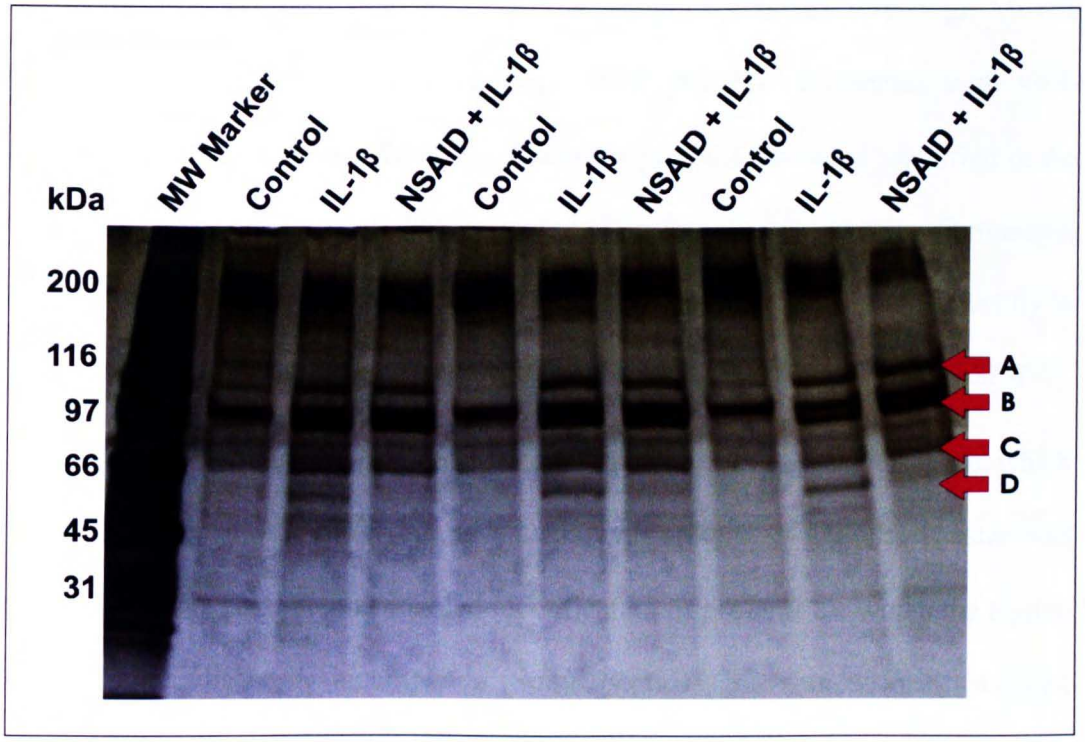

Figure 49. Electrophoretic protein profiles of medium from cartilage explants incubated alone (control), or treated with equine interleukin-1beta (IL-1β), and a nonsteroidal anti-inflammatory drug (NSAID + IL-1β)

Silver staining of the one-dimensional (1-D) gel identified stronger bands in the IL-1β and NSAID-treated samples compared to controls at roughly 110 kilodaltons (kDa) (arrow A), 90kDa (arrow B) and 66kDa (arrow C). Arrow D highlights a band around 55kDa present in IL-1β-treated samples, but not in control or IL-1β plus NSAID-treated samples.

Identification of Proteins in the Sample Media

The majority of secreted proteins identified using LC-MS/MS with high Mowse scores in multiple samples were cartilage ECM proteins or proteins with well-established matrix functions (table 3). Abundant proteins that were identified in the majority of samples from all horses included aggrecan core protein, fibronectin, decorin and COMP. The database search identified some proteins more frequently in IL-1β-treated samples compared to untreated controls, namely MMP-3 and TSP-1.

Cross-species matching compares the protein sequence entries in the database, which exhibit the closest homology to the 'unknown' protein of interest (Lester and Hubbard, 2002). This method enabled identification of proteins for which the equine versions were absent or incomplete. Some peptides, such as decorin, matched proteins from multiple species hits (human, horse, cow, rat, dog and rabbit), and where the horse sequence was present in the database, it generally achieved the higher Mascott Mowse score compared to that of the closest homologous sequence in the other species. The only exception was fibronectin, which achieved a top Mascott Mowse score of 642 with the bovine sequence but only 221 with the equine sequence. This could be due to the entry in the equine database containing an error, which is possible when a protein sequence has been translated directly from DNA (Barrett *et al.*, 2005). It could also be due to there being two variants of this protein in vertebrates (insoluble found in the ECM and soluble found in the plasma), the one currently present in the equine database may not have been detected, hence the greater homology with bovine. Where the horse sequence was absent from the database, multiple species matching increased confidence in the protein detected, for example, aggrecan core protein which had peptide matches with human, mouse, rat,

pig and dog. Some peptides only matched proteins from one species such as equine MMP-3, or two species such as equine and bovine MMP-1.

The majority of proteins identified in this study were ECM components or ECM-associated, however, a small number of additional proteins were also found. Eleven non-ECM proteins were present in multiple samples and are shown in table 4. Many of these had lower Mowse scores under 55, except serum albumin precursor with a score of 71. Peptides with lower Mowse scores mean they are more likely to be random matches than real matches, but the automatic threshold score above which matches were considered real was set at a score of 40. Peptides with fragment ion matches under this threshold were manually checked to determine their accuracy.

Many proteins were only identified in a single sample from the 54 samples tested. Table 5 details peptides from these proteins that contained four or more continuous fragment ions in a series. The presence of four or more fragment ions in a row was defined as the criterion for which a single peptide match was considered to be a true positive, rather than five fragment ions as is standard. This was because some of the peptides were small and only consisted of five or six amino acids. Therefore, as detecting all ions is unlikely, the detection of four ions in a row was set as the limit confirming the peptide match. The presence of some of the proteins in table 5 may be due to contamination, for example keratin from hair, skin or nails during tissue collection, culture or sample processing (Chamrad *et al.*, 2003). Table 6 shows the proteins found in only one sample which have peptides with fewer than 4 continuous fragment ions in a series. These proteins have low Mowse scores of 55 or less.

Protein name	Species matched	Accession number	Entry name	Mascot Mowse score	Sequence Coverage (%)	No. of matched peptides	Function
Fibronectin	Horse, Mouse, Cow, Rat	P07589	FINC_BOVIN	642	9	16	ECM structure, interacts with cells + collagen
Cartilage Oligomeric Matrix Protein	Mouse, Human, Cow, Rat	Q9R0G6	COMP_MOUSE	554	16	11	ECM structure, interacts with collagen, fibronectin, integrins
Matrix-Metalloproteinase-3	Horse	Q28397	MMP3_HORSE	366	19	11	ECM degradation: fibronectin, collagen, gelatins, proteoglycans
Aggrecan Core Protein	Human, Mouse, Rat, Pig, Dog	P16112	PGCA_HUMAN	353	3	8	Compression resistance in ECM Interacts with COMP
Clusterin	Horse, Dog	Q29482	CLUS_HORSE	318	14	5	Secreted glycoprotein, extracellular molecular chaperone activity + apoptosis regulation
Thrombospondin-1	Human, Mouse, Cow	P07996	TSP1_HUMAN	227	5	6	Adhesive glycoprotein for cell-cell + cell-matrix interactions
Matrix-Metalloproteinase-1	Horse, Cow	Q9XSZ5	MMP1_HORSE	227	10	5	ECM degradation: collagens
Fibronectin	Horse, Mouse, Cow, Rat	Q28377	FINC_HORSE	221	11	3	
Chondroadherin	Human, Mouse, Cow, Rat	O15335	CHAD_HUMAN	220	13	4	Chondrocyte attachment via integrins Interacts with collagen type II
Biglycan	Horse, Cow	O46403	PGS1_HORSE	183	13	4	Collagen fibre assembly
Decorin	Horse, Human, Cow, Rat, Dog, Rabbit	O46542	PGS2_HORSE	156	10	3	Fibril formation in ECM, binds to collagen I, II, fibronectin, TGF-β
Cartilage Intermediate Layer Protein-1	Human, Mouse, Pig	O75339	CILP1_HUMAN	99	2	3	ECM organisation (scaffolding) Antagonises TGFβ1 and IGF-1 function
Prolargin precursor	Human, Mouse	P51888	PRELP_HUMAN	96	3	1	Anchors basement membranes Binds collagens and proteoglycan
TNF receptor 11B precursor	Mouse	O08712	TR11B_MOUSE	87	3	1	TNF receptor superfamily member 11B Promotes osteoclast apoptosis
Fibromodulin	Rat, Human, Mouse	P50609	FMOD_RAT	82	8	3	Fibril formation in ECM, binds to collagen I, II

Table 3. Secreted extracellular matrix proteins and proteolytic enzymes present in multiple samples identified using the SwissProt 51.6 database

Protein identification using cross species matching often found matches with multiple species. Horse sequence-matched proteins normally achieved the top Mowse score out of all species matched for that protein, except for fibronectin (grey shaded rows), which had a higher Mowse score for the bovine sequence than the horse sequence. Proteins listed in order of Mowse score (highest score first).

Protein name	Species matched	Accession Number	Entry name	Mascot Mowse score	Sequence Coverage (%)	No. of matched peptides	Function
Vimentin	Cow, Green monkey	P48616	VIME_BOVIN	301	12	6	Intermediate filament protein part of cellular cytoskeleton
Trypsin	Pig	P00761	TRYP_PIG	134	16	3	Used to digest protein
Serum amyloid A protein	Horse, Cow,	P19857	SAA_HORSE	123	25	3	Apolipoprotein secreted into plasma in response to cytokines
Alpha Enolase	Cow, Human	Q9XSJ4	ENOA_BOVIN	94	7	4	Intracellular metabolic enzyme role in glycolysis and hypoxic tolerance
Serum amyloid A-2 protein	American mink	P02739	SAA2_MUSVI	74	12	2	Apolipoprotein secreted into plasma in response to cytokines
Transcription termination factor 1	Human	Q15361	TTF1_HUMAN	68	2	3	Nuclear protein terminating transcription
Serum albumin	Cow	P02769	ALBU_BOVIN	71	1	1	Plasma protein
Breast cancer type 2 protein susceptibility homolog	Cat	Q864S8	BRCA2_FELCA	51	0	3	Involved in double strand break repair
Lysozyme C, kidney isozyme	Sheep	P80190	LYSCK_SHEEP	47	6	1	Bacteriolytic function
Sodium/hydrogen exchanger 2	Rabbit	P50482	SL9A2_RABIT	45	1	1	Membrane protein involved in pH regulation to eliminate acids generated by metabolism
Cytoplasmic dynein 1 heavy chain 1	Human	Q14204	DYHC1_HUMAN	44	0	1	Organelle and vesicle motility along microtubules

Table 4. Non-extracellular matrix proteins identified in six or more samples identified using the SwissProt 51.6 database

Protein identification using cross species matching often found matches with multiple species. Horse sequence-matched proteins normally achieved the top Mowse score out of all species matched for that protein. Table lists the non-extracellular matrix (ECM) proteins in order of Mowse score (highest score first).

Protein name	Species matched	Accession Number	Entry name	Mascot Mowse score	Sequence Coverage (%)	No. of matched peptides	No. of continuous fragment ions in a series	Function
Keratin, type II cytoskeletal 1	Human	P04264	K2C1_HUMAN	87	3	2	9	Hair/ skin contaminant
Triosephosphate isomerase	Dog	P54714	TPIS_CANFA	52	4	1	9	Glycolytic enzyme
Fc receptor-like A	Mouse	Q920A9	FCRLA_MOUSE	49	1	1	5	Cytoplasmic proteins resembling Fc receptors
Glucose-6-phosphate 1-dehydrogenase 2	Mouse	P97324	G6PD2_MOUSE	45	3	2	4	Cytosolic enzyme involved in NAPDH/ glutathione protection against oxidative damage
Centromere protein C1	Mouse	P49452	CENPC_MOUSE	42	0	1	4	Nuclear protein apoptotic cells
Lactadherin	Pig	P79385	MFGM_PIG	40	2	1	4	Secreted membrane protein involved in phagocytic removal of apoptotic cells
Max-like protein X	Human	Q9UH92	MLX_HUMAN	38	2	1	4	Nuclear and cytoplasmic isoforms – regulate transcription

Table 5. Non-extracellular matrix proteins identified in only one sample, with four or more continuous fragment ions

Identifications of proteins using cross species matching often found matches with multiple species. The identifications of these proteins relied on peptides with four or more continuous fragment ion matches using the SwissProt 51.6 database. Proteins listed in order of Mowse score (highest score first).

Protein name	Species matched	Accession number	Entry name	Mascot Mowse score	Sequence Coverage (%)	No. of matched peptides	Function
DNA-dependent protein kinase catalytic subunit	Dog	Q8WN22	PRKDC_CANFA	55	0	3	Nuclear protein, molecular sensor for DNA damage
Serine-protein kinase ATM	Mouse	Q62388	ATM_MOUSE	55	0	3	Nuclear /cytoplasmic vesicle protein, molecular sensor for DNA damage
Histone acetyltransferase	Human	Q8WYB5	MYST4_HUMAN	53	1	2	Nuclear protein, involved in transcriptional activation and repression
Bromodomain and PHD finger-containing protein 3	Human	Q9ULD4	BRPF3_HUMAN	51	2	3	Nuclear protein, component of complex with MYST4 activity
Ubiquitin carboxyl-terminal hydrolase 24	Human	Q9UPU5	UBP24_HUMAN	50	0	2	Involved in the ubiquitin-dependent proteolytic pathway
Putative pre-mRNA-splicing factor ATP dependent RNA helicase DXH15	Human	O43143	DHX15_HUMAN	48	2	2	Nuclear protein, pre-mRNA processing factor involved in disassembly of spliceosomes
Nuclear mitotic apparatus protein 1	Human	Q14980	NUMA1_HUMAN	48	0	2	Nuclear chromosome protein, structural component
Bullous pemphigoid antigen 1	Human	Q03001	BPA1_HUMAN	47	0	2	Cytoplasmic protein, a cytoskeletal linker protein
Sodium/calcium exchanger 1	Cow	P48765	NAC1_BOVIN	47	1	2	Cell membrane protein, transports Ca out during relaxation to prevent intracellular stores overloading
Thyroid receptor-interacting protein-11	Human	Q15643	TRIPB_HUMAN	47	0	1	Golgi, cytoplasmic and peripheral membrane protein, enhances thyroid receptor-modulated transcription
Plectin-1	Rat	P30427	PLEC1_RAT	46	0	3	Cytoplasmic protein, involved in cross-linking and stabilisation of cytoskeletal intermediate filament
Bcl-2/adenovirus E1B 19kDa-interacting protein 2-like protein	Mouse	Q99JU7	BNIPL_MOUSE	45	6	1	Nuclear and cytoplasmic protein bridging BCL2 and ARRHGAP1/CDC42 in promoting cell death

Table 6. Non-extracellular matrix proteins identified in one sample with fewer than four continuous fragment ion matches

Identifications of proteins using cross species matching often found matches with multiple species. The identifications of these proteins relied on peptides with less than four continuous fragment ion matches using the SwissProt 51.6 database. Proteins listed in order of Mowse score (highest score first). *Continued on next page.*

Protein name	Species matched	Accession number	Entry name	Mascot Mowse score	Sequence Coverage (%)	No. of matched peptides	Function
Epidermal growth factor receptor kinase substrate 8-like protein 3	Human	Q8TE67	ES8L3_HUMAN	44	2	2	Cytoplasmic protein, interacts with ABI1 to regulate cell growth and Erk pathway activation
Glutaminyl-tRNA synthetase	Human	P47897	SYQ_HUMAN	44	2	2	Cytoplasmic protein, involved in catalysing reaction for glutamate metabolism, and ATP->ADP
Integrin beta-5	Human	P18084	ITB5_HUMAN	43	1	2	Membrane protein, receptor for fibronectin
Glial fibrillary acidic protein	Cow	Q28115	GFAP_BOVIN	43	5	2	Cytoplasmic protein, a cell-specific marker distinguishes astrocytes from other glial cells
Phosphoglycerate kinase 1	Chinese Hamster	P50310	PGK1_CRIGR	43	2	1	Cytoplasmic glycolytic enzyme
Synergin gamma (AP1 subunit gamma-binding protein 1)	Mouse	Q5SV85	SYNRG_MOUSE	42	1	2	Cytoplasmic protein involved in endocytosis and membrane trafficking at the trans-Golgi network
Beta, beta carotene 9',10'-oxygenase	Crab eating macaque	Q8HXG8	BCDO2_MACFA	42	3	2	Oxidatively cleaves beta-carotene and lycopene
Death-associated protein kinase 3	Rat	O88764	DAPK3_RAT	41	2	1	Nuclear + cytoplasmic protein, positively regulates apoptosis
Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	Cow	O77784	IDH3B_BOVIN	41	1	1	Mitochondrial enzyme involved in the citric acid cycle by oxidative decarboxylation of isocitrate
Cytosolic phospholipase A2 delta	Mouse	Q50L43	PA24D_MOUSE	40	2	2	Cytoplasmic protein that hydrolyses glycerophospholipids
Protein arginine deiminase type-4	Human	Q9UM07	PADI4_HUMAN	39	1	2	Cytoplasmic + nuclear protein, catalyses citrullination/ deimination of arginine residues of proteins

Table 7. continued. Non-extracellular matrix proteins identified in one sample with fewer than four continuous fragment ion matches

Identifications of proteins using cross species matching often found matches with multiple species. The identifications of these proteins relied on peptides with less than four continuous fragment ion matches using the SwissProt 51.6 database. Proteins listed in order of Mowse score (highest score first).

Quantitative Western Blotting Confirms Identity of Several Proteins as Predicted by the SwissProt 51.6 Database

To validate some of the putative identifications, quantitative western blotting was used to confirm the presence of selected matrix associated proteins and quantify the relative intensities of the protein bands in the IL-1β-treated and IL-1β plus NSAID-treated samples versus the untreated controls (figure 50). Western blotting confirmed the presence of CILP-1 (60kDa), clusterin (39kDa) and its precursor form (70kDa) and MMP-3 (54kDa) in the samples. Three proteins had a different observed molecular weight (MW) compared to their predicted weights: MMP-1 (observed MW ~102kDa, predicted MW = 54kDa), TSP-1 (observed MW ~125kDa, predicted MW = 165-198kDa) and TSP (observed MW ~125kDa, predicted MW = 170-180kDa).

IL-1β-treated samples showed increased protein levels of MMP-1, MMP-3 and TSP-1 in comparison to controls, which were decreased with the addition of the NSAID.

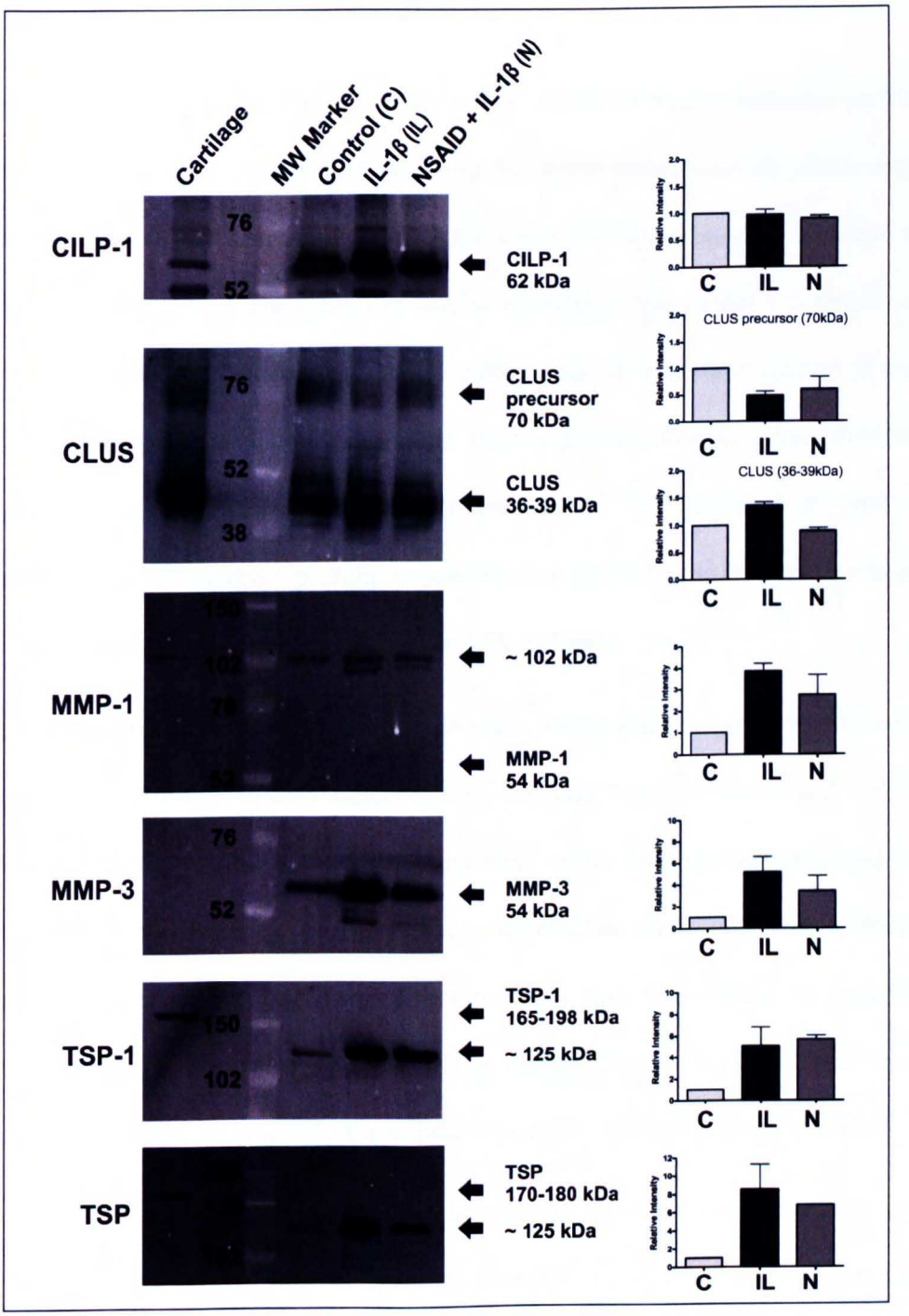

Figure 50. Western blot quantification of proteins in media from cartilage explants alone (control), and with equine interleukin-1beta (IL-1β), and the nonsteroidal anti-inflammatory drug, carprofen (NSAID + IL-1β) combined

Blotting for cartilage intermediate layer protein-1 (CILP-1), clusterin (CLUS), matrix metalloproteinase (MMP)-1, MMP-3 and thrombospondin (TSP)-1. Arrows labelled with the protein name indicate the predicted molecular weight (MW) in kilo Daltons (kDa), arrows with no protein name indicate the observed MW.

5.4. Discussion

This study set out to refine a serum-free explant model of equine articular cartilage and test the feasibility of using it for studying the major proteins in the secretome, as well as changes in the secretome in response to a pro-inflammatory mediator and anti-inflammatory drug. The intention was to investigate the model's potential as a screening system for identifying potential biomarkers of structural change in early OA. High-throughput proteomics and cross species peptide matching were then used to identify the main proteins present in the secretome. This approach is rapid and invaluable for investigating protein expression in animal species that have few protein sequences in the databases (Liska and Shevchenko, 2003).

Using comparative proteomic analysis, this study identified the presence of several expected proteins in the supernatants of equine cartilage explants stimulated with IL-1β. A number of abundant secreted proteins involved in cartilage structure and thus constitutively expressed by cartilage were also identified, such as aggrecan, fibromodulin, decorin and biglycan. These proteins have been found in proteomic studies of cartilage from other species such as human (Wu *et al.*, 2007) and mouse (Wilson *et al.*, 2008), and thus aid the validation of this equine cartilage model.

One of the most abundant proteins in the explant system was COMP, a non-collagenous matrix protein that mediates chondrocyte attachment to the matrix (Di Cesare *et al.*, 1994) and organises matrix assembly (Rosenberg *et al.*, 1998). COMP has been found in the synovial fluid, serum and urine from human and equine OA patients, and its correlation with disease severity and progression has led to it being suggested as a potential biomarker for OA (Lohmander *et al.*, 1994; Petersson *et al.*, 1998; Clark *et al.*, 1999; Arai *et al.*, 2005; Misumi *et al.*, 2006; Tseng *et al.*, 2009). This suggests that COMP may be a useful marker of OA. The presence of COMP in

control and treated samples from this model concurs with that of other *in vitro* studies, which have found abundant COMP in the media of cartilage explants (Wu *et al.*, 2007; Stevens *et al.*, 2008; Wilson *et al.*, 2008). Therefore, although further work is needed to determine levels of COMP in the model, these initial findings support the use of the model for testing clinically defined markers.

IL-1β-treatment always resulted in equine MMP-3 being detected in the secretome, in comparison to being detected in only one of the 18 untreated control samples. MMP-3 is a proteolytic enzyme that degrades ECM components such as fibronectin, collagens and cartilage proteoglycans. Its protein levels are increased in equine clinical OA cases and its gene expression up-regulated in IL-1β-stimulated chondrocytes *in vitro* (Brama *et al.*, 2000c; Tung *et al.*, 2002). Immunolocalisation studies have located MMP-3 in the synovium, cartilage and medium of cultured cartilage explants from OA patients (Okada *et al.*, 1992). The proteomic data suggested that MMP-3 was significantly higher in IL-1β-treated samples compared to controls. However, this required confirmation using a quantitative method. Therefore, western blot analysis of the samples was used to confirm the presence of MMP-3 and demonstrate that levels of this protein were higher in IL-1β-treated cartilage explants in comparison to controls. Moreover, the level of this proteolytic enzyme was lower in the NSAID-treated samples. The weak band(s) in the tissue homogenate controls showed that MMP-3 was present in cartilage, but in lower concentrations, than in the IL-1β-treated samples and thus may have been below the detection threshold used to analyse the samples. However, both the LC-MS/MS and western blot data confirm that equine articular cartilage explants also secrete MMP-3 in response to equine IL-1β. Interestingly, a recent study investigating the secretome of human articular explants and chondrocytes in culture using stable isotope labelling

with amino acids in cell culture (SILAC) found that MMP-3 was actively synthesised and released in both culture systems (Polacek *et al.*, 2010). These authors used macroscopically healthy cartilage, suggesting that the results differ from those found in this thesis where MMP-3 was predominantly found in the media of IL-1β-stimulated cartilage, rather than unstimulated controls. However, despite the fact the cartilage was macroscopically healthy, it was obtained from patients with moderate to advanced knee cartilage defects. Therefore, although the cartilage used by Polacek *et al.* (2010) looked healthy, the fact that it was obtained from diseased joints suggests that it was not, as it could have been exposed to various pathological stimuli such as IL-1β. In fact, a study has shown that macroscopically healthy cartilage taken from joints with OA lesions commonly have microscopic changes associated with OA, such as increased presence of IL-1β in the superficial and middle cartilage zones, and higher glycosaminoglycan loss compared to lesion-free joints (Weaver *et al.*, 2006). Consequently, the work of Polacek *et al.* (2010) appears to concur with the findings of this chapter, namely that proteomic techniques have confimed that MMP-3 is secreted in response to the degenerative stimulus, IL-1β. The results of this study therefore add further support to the work in chapter 2 that the explant model is a model of early OA.

Positive cross species matching for TSP-1 was identified with bovine, human and mouse. The equine sequence for TSP-1 was not present in the SwissProt database at the time of this study. However, the fragments matched the protein motifs of TSP-1 from multiple other species, thus increasing our confidence in the presence of this protein in the cartilage secretome. TSP-1 is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions (Lawler and Hynes, 1986; Chen *et al.*, 2000). Human cartilage studies have shown that TSP-1 is increased in early OA

cartilage lesions (Pfander *et al.*, 2000) and the proteomic and western blot results from this study, suggest that this is reflected in the cartilage explant model of early equine OA. Although the cartilage positive control produced a band at about 170kDa (within the range of the antibody manufacturers' predicted molecular weight) with two TSP-1 specific antibodies from different companies, the observed bands in the secretome samples were roughly 40kDa lower with a molecular weight at approximately 125-130kDa. TSP-1 is reported in the literature as a 420-480kDa protein with 180kDa subunits, that are revealed on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) gels under reducing conditions (Di Cesare *et al.*, 1994). Calculation of the predicted protein mass for TSP-1_HUMAN using the expasy tools program (http://www.expasy.org/tools/peptide_mass.html) revealed that the full length sequence has a predicted protein mass of 129.4kDa and a predicted protein mass after signal peptide cleavage of 127.5kDa. Post-translational cleavage by signal peptidases are responsible for the proteolytic processing of protein precursors as they leave the cell, thereby reducing the molecular weight of the protein (Dalbey *et al.*, 1997). The observed molecular weight of TSP-1 in the secretome samples corresponds well with the predicted protein mass after signal peptide cleavage from the amino acid sequence in the database. This suggests that TSP-1 may have been post-translationally cleaved. However, the cartilage control still had a higher molecular weight than predicted. Work using epithelial cells has shown that despite an expected molecular weight of approximately 130kDa, TSP-1 migrates with a molecular mass between 160 and 180kDa on traditional SDS-PAGE gels (O'Rourke *et al.*, 1992), which corresponds to the weight seen in the cartilage tissue. Although the cartilage tissue was run on a Bis/Tris gel in an SDS-PAGE system, aberrant migration may be responsible for the discrepancy in predicted and

observed molecular weight. However, the SwissProt database entry for TSP-1 infers that it is associated with the external side of the plasma membrane. Thus, it may require cleavage off the membrane to be present in the secretome. The unmodified C-terminal domain for TSP-1 is 27.9kDa, which when subtracted from the observed secretome mass (170kDa), results in a band of 142.1kDa, nearing the observed molecular weight of TSP-1 in the secretome samples. Thus aberrant migration or cleavage of TSP-1 from the membrane seem the most likely explanations for the differences.

Western blotting for MMP-1 produced an observed band of roughly 102kDa in both the cartilage control and all the secretome samples (present as a double band in IL-1β-treated samples), which was nearly double the predicted molecular weight (54kDa). The MMP-1 sequence has the potential to form a double bond by virtue of possessing more than one cysteine residue in its amino acid sequence, and although the samples were run under reducing conditions, it may be that the observed band is a dimer that is resistant to the reducing agent. However, there are few published reports of a dimer form of MMP-1 (Iyer *et al.*, 2006). Post-translational modifications such as phosphorylation and glycosylation can lead to a mass increment relative to the calculated molecular weight of proteins, but these are not generally detectable on 1-D gels (Jensen, 2004). However, there are no reports of these modifications in the published literature or in the uniprot database for MMP1_HORSE. Therefore, it may be that the MMP-1 band is due to the dimer being present or non-specific binding. Further studies using human cartilage as a positive control are neded to ensure that the antibody is working correctly. After this, several sample preparations incubating with reducing agent for different lengths

of time and at different temperatures can be conducted to try and prevent dimerisation.

The abundance of cartilage ECM-associated proteins in the top scoring entries highlights the specificity of this model for studying the secretome. However, despite using a targeted approach and an explant culture system, a small number of non-matrix associated proteins were also identified in the media of several samples, for example, vimentin, dynein heavy chain, alpha enolase and sodium hydrogen exchanger 2 (see table 4). There are a number of possible explanations and interpretations for the presence of these proteins. The obvious explanation for the presence of these non-ECM proteins is cell death; the detection of these proteins may be attributable to dead or dying chondrocytes. However, it is known that there are frequently observed proteins in many proteomics experiments regardless of species or tissue being studied, such as alpha enolase and vimentin (Petrak *et al.*, 2008; Wang *et al.*, 2009). Thus, whilst these proteins may reflect a commonly observed cellular stress response between tissues, it must be considered that they may simply represent a limitation of the method.

In addition to the cartilage ECM and chondrocyte-associated proteins, the database search also detected some seemingly unlikely candidates for biomarkers of arthritis. For example, breast cancer type 2 protein susceptibility homolog, which is the protein encoded the tumour suppressor gene, BRCA2. However, this protein is involved in DNA repair, namely double-strand break repair and homologous recombination (O'Donovan and Livingston, 2010). DNA damage is found in OA articular chondrocytes, and in non-OA chondrocytes where oxidative strand breaks are induced by NO produced from culturing with IL-1α (Davies *et al.*, 2008). Thus, oxidative damage is involved in OA and it may be that breast cancer type 2 protein

susceptibility homolog is involved in the chondrocyte repair response, and released into the media from dying cells.

It is also highly likely that some of these less abundant proteins with lower Mowse scores could be proteins of interest in their own right or modifications of the abundant proteins, which are relevant to OA. Ideal biomarkers for OA are those that can indicate the very early stages of disease before too much destruction has occurred, therefore they are unlikely to be fragments or degraded forms of ECM proteins (Mobasheri and Henrotin, 2010). Thus, although the method used a Mowse score of 40 as the cut-off point, it could be that proteins just under the detection threshold have the most useful potential as biomarkers. For example, protein arginine deiminase type-4 (PADI4) which had a Mowse score of 39 in table 6, is known to catalyse citrullination of arginine residues of proteins. Citrullinated peptides can act as autoantigens, thereby increasing the risk of RA (Vossenaar *et al.*, 2004). As such, PADI4 gene expression has been detected in the synovial tissue from RA patients (Suzuki *et al.*, 2003). In addition, autoantibodies against PADI4 are associated with increased RA severity (Harris *et al.*, 2008). Although RA is an autoimmune disease, unlike OA, the protein may have other functions and potential involvement in OA. The presence of PADI4 in the supernatant of the cartilage explants in this study may partly explain why this enzyme could be a target of autoantibodies to citrullinated proteins; in fact, this antigen may not be as specific for RA as previously suggested (Auger *et al.*, 2010; Kolfenbach *et al.*, 2010), the enzyme may also be released in OA. PADI4 is one of the many less abundant proteins identified in the model that may be potential biomarkers. More studies with larger sample numbers should provide more information in this area. However, greater potential lies in using methods that facilitate mining below the abundant

proteins in the secretome to allow robust and clear detection of the lower abundance components. The most obvious of these methods would be the depletion of the major/ high abundance proteins, which is commonly used when studying biological fluids such as plasma and cerebrospinal fluid (Tirumalai *et al.*, 2003; Shores and Knapp, 2007). Another approach would be the use of metabolic labelling during culture to allow the newly synthesised proteins to be studied (Zwickl *et al.*, 2005). Adopting these approaches will aid identification of these less abundant but potentially more interesting proteins.

Proteomics has been used to study soluble extracts of cartilage (Wu *et al.*, 2007; Guo *et al.*, 2008), chondrocyte whole cell lysates (Ruiz-Romero *et al.*, 2008), synovial fluid (Gobezie *et al.*, 2007) and synovial fibroblasts (Bo *et al.*, 2009), from both normal and OA patients. However, using cartilage and chondrocytes from OA patients raises a number of complications. Firstly, it can be difficult to obtain appropriate controls, using healthy joints from one group of patients as a control does not address individual variation. Similarly using control cartilage from a healthy corresponding joint of an OA patient, is invasive, and may not be an appropriate control due to altered use of the non-affected limb. Secondly, early OA clinical samples can be difficult to obtain, because the disease can be subclinical in the early stages and conventional radiography (the standard test for assessing joint space narrowing), has limited sensitivity (Chan *et al.*, 1991). Consequently, many explant studies use cartilage taken whilst the patient is undergoing corrective surgery such as joint replacement (Uitterlinden *et al.*, 2006; Attur *et al.*, 2008). Thus, many clinical samples would be from the more advanced stages of OA. The cartilage explant model uses macroscopically and microscopically healthy cartilage from the same animal, allowing the same cartilage to be treated both as a control and with

inflammatory stimuli, such as cytokines, to induce degradation in the explants. Therefore, the model can reduce some of the confounding variables associated with clinical OA samples such as individual variation, differing disease aetiology and the stage of disease progression. In effect, using the cartilage explant system provides a more homogenous model with appropriate controls. In addition, explants can be cultured in serum-free medium, which removes the issue of contaminating serum proteins binding to the proteins and preventing the identification of the potentially more interesting lower abundance proteins.

The absence of fibrillar and non-fibrillar collagens in the secretome supports the existing evidence that the model reflects early events in OA, before any major structural changes occur in the fibrillar matrix. Increased collagen type II denaturation and loss from the cartilage is seen in OA (Hollander *et al.*, 1994). In juvenile bovine cartilage explants stimulated with IL-1α and OSM, collagen loss is not observed until after 22 days in culture, an effect less evident in older cartilage (Blain *et al.*, 2010). Thus, suggesting that the proteins identified in the secretome preceed the degradation and release of collagens and their fragments, thereby supporting the use of the model as an early model of OA.

The advantage of a proteomic approach over a genomic approach to studying potential disease indicators are that gene expression levels do not necessarily correlate with protein levels (Nie *et al.*, 2007). Clusterin mRNA expression levels are increased in early OA cartilage compared to control (Connor *et al.*, 2001), whereas IL-1α-stimulated cartilage explants show decreased levels of clusterin protein compared to control cartilage (Wilson *et al.*, 2008). This could reflect the differences between clinical cases and *in vitro* models of OA, the stage of OA being studied and the various stimuli used to initiate cartilage degradation. However,

whilst further *in vivo* work needs to be performed, to determine levels of proteins in the synovial fluid and serum of clinical cases, *in vitro* studies looking at proteins rather than gene expression are invaluable prerequisites.

This chapter has used the existing explant model of equine articular cartilage and adapted it for subsequent high-throughput proteomic work. One of the key advantages of this cartilage explant model is that it is serum-free. Therefore, the problem of contaminating proteins such as albumins and immunoglobulins, which make up a large percentage of serum, is eliminated from this approach. Another advantage of this culture system is that the resident cells are maintained in their native microenvironment overcoming the difficulties and challenges associated with chondrocyte de-differentiation in proteomic studies that use cultured and passaged cells *in vitro*. Using cross species peptide matching, a number of relevant proteins with well-established ECM functions in cartilage including: cell-matrix and matrix-matrix interactions (fibronectin, TSP-1, COMP, CHAD); matrix turnover (MMP-1, MMP-3) and extracellular molecular chaperone activity (clusterin) were identified. Other proteomic studies using cartilage from other species have identified similar panels of extracellular proteins (Wu *et al.*, 2007; Stevens *et al.*, 2008; Wilson *et al.*, 2008). The similarities between the list of proteins identified in this study and those published by other laboratories increase confidence in these data and justifies the application of high-throughput techniques in cartilage research. This chapter has highlighted the presence of increased MMP-3 and TSP-1 in the supernatants of equine cartilage explants stimulated with equine IL-1β. These proteins may be involved in early responses to pro-inflammatory cytokines in cartilage and may be potentially useful biomarkers for the detection of early changes in OA. The explant culture system employed facilitated a rapid analysis of secreted proteins in a cartilage

model that may aid the discovery of OA biomarkers. It may be that a combination of several of these biomarkers can improve accuracy in the diagnosis and prognosis of OA (Williams, 2009). Future studies will exploit this serum-free model to investigate the effects of other pathophysiologically relevant stimuli such as mechanical injury (Quinn *et al.*, 1998) hypoxia and reoxygenation (Cernanec *et al.*, 2002) and acidic pH (Schwartz *et al.*, 1976).

In conclusion, the work described here has highlighted a novel adaptation of this model. Although further research is needed, the promising results of these preliminary studies suggest that this high-throughput technique may help identify proteins, alone or in combination, that may be modulated by plant extracts in the model as well as having the potential to aid novel biomarker discovery. The detection of suitable biomarkers that can be quantified, validated and qualified *in vitro* and *in vivo*, would enable faster screening and evaluations of potential non-protein nutraceuticals for supporting joint health in both humans and companion animals. Thus, this chapter describes a new method that may aid nutritional targeting of inflammatory pathways and catabolic mediators involved in equine OA.

CHAPTER 6. PILOT FIELD TRIAL OF A PLANT EXTRACT COCKTAIL

6.1. Introduction

Whilst work for the previous chapter was being undertaken, the opportunity to run a field-based trial arose. This was a useful chance to gain experience in designing and managing field trials, as well as determining the *in vivo* effects of the materials tested in chapter 4. The trial was set up to replicate a scenario of feeding joint health products to horses with chronic stiffness issues, and to judge efficacy as an owner would, i.e. a visibly detectable clinical improvement in mobility.

The work in chapter 4 determined that non-cytotoxic concentrations of PT significantly decreased IL-1β-stimulated GAG and PGE_2 release from cartilage explants in the *in vitro* model. In the joint supplement industry, *in vitro* data such as these, are often extrapolated and translated into *in vivo* efficacy with limited, if any, supporting *in vivo* data. It is well recognised that despite the abundance of nutraceuticals on the market for human consumption purporting to improve joint mobility, there is a paucity of good quality, unbiased *in vivo* trials to support their claims (McAlindon *et al.*, 2000). Many equine *in vivo* studies are poorly designed, either in numbers, lack of appropriate controls, assessor blinding or treatment cross-over (Pearson and Lindinger, 2009). Therefore, *in vitro* studies may aid the discovery of novel actives, alone or in combination, for joint supporting joint health, that can be followed up by clinical or field trials to determine their effects *in vivo*. In addition, *in vitro* studies may identify the potential modes of action that may operate *in vivo* to cause a beneficial effect.

Animal trials lack the verbal input from their subjects from which to evaluate efficacy. Human subjects can inform the assessors of a perceived improvement in pain, stiffness or general well-being, but their opinion may be influenced by other people or the placebo effect. Therefore, different methods of assessment are required for animal and human trials. Previous equine trials have used standardised lameness scoring systems for detecting an improvement in response to drugs (Sabate *et al.*, 2009) and supplements (Hanson *et al.*, 1997). However, the horses selected for this trial were not lame; instead, they were bilaterally stiff. Stiffness is defined as 1. not easily bent; rigid or half way to rigidity, tetany, 2. not moving freely, 3. being unable to move easily without pain (Blood and Studdert, 1999; Soanes, 2000). For the purposes of this thesis, stiffness is defined as a restricted range of motion in the hindlimb joints. There is no standardised scoring system for stiffness, therefore, a scoring system was developed by the assessor to detect changes in the severity of stiffness over time. Stiffness was selected rather than lameness, because the aim of the trial was to replicate a scenario in the field where owners feed joint supplements to stiff horses to see if they have a beneficial effect, sometimes before seeking medical advice. In addition, the target horse population was that of stiff horses that did not require medication. It was not possible to find a large group of lame horses that were not on medication.

This study used both a commercially available joint health product and a formulated cocktail of plant extracts previously tested in an *in vitro* model of OA (chapter 4) in a field-based trial. The objective was to determine whether either supplement could improve mobility in a group of horses with chronic, low-grade hindlimb stiffness over a six-week period. The hypothesis was that the PT and CP would significantly reduce stiffness in the horses.

6.2. Material and Methods

Supplements

The supplements in the trial were the same materials as described in chapter 4. The plant extract cocktail (PT), the commercial joint health product Cortaflex (CP) and a placebo (PL) of alfalfa meal. Supplements were provided to the farms in identical white containers, labelled A, B and C, the contents of which were unknown to the assessor and staff.

Subjects

Thirty-three horses and ponies in permanent care of Redwings horse sanctuary in Norfolk were non-randomly selected for the trial as they needed to meet the following inclusion criteria: 1. chronic stiffness, defined as bilateral low-grade stiffness and clinically presented as reduced or restricted flexion and movement in hindlimbs, for over three months; 2. healthy horse or pony of any gender, breed, age and weight; 3. not receiving medication for musculoskeletal conditions. Exclusion criteria were as follows: 1. serious co-existing condition; 2. has been on long-term, or is currently receiving, NSAID or corticosteroid therapy.

The number of horses for statistical power (n=11 per group) was based on a previous study that had used a similar stiffness assessment and shown significance at $p<0.05$ with n=9 per group in a two-way cross-over trial (Dyson *et al.*, 2001).

Ages ranged from 4-25 years (mean 16.1 years) and height ranged from 9hh to 17hh (median 13hh). The ratio of horses under 400kg to over 400kg was 21:12. The ratio of mares to geldings was 16:17 (table 7).

Location	Horse ID	Age (yrs)	Height (hh)	Sex	Colour	Breed	Weight
Hapton	1	15	12	Gelding	Bay	Native X	<400kg
	2	18	12	Gelding	Bay	Native X	<400kg
	3	18	14	Gelding	Bay	New Forest X	>400kg
	4	13	15	Mare	Chestnut	Thoroughbred X	>400kg
	5	10	14	Mare	Chestnut	Arab	>400kg
	6	20	15	Gelding	Bay	X breed	>400kg
	7	16	13	Gelding	Bay	Welsh Section D	<400kg
	8	20	14.2	Gelding	Mixed	Welsh X	<400kg
	9	16	12	Mare	Grey	Welsh X	<400kg
	10	16	14	Gelding	Chestnut	Welsh Section D	>400kg
	11	20	14	Gelding	Bay	X breed	>400kg
Piggots	12	11	13	Mare	Grey	Connemara	<400kg
	13	23	16	Gelding	Bay	Hunter	>400kg
	14	20	15	Gelding	Chestnut	Thoroughbred	>400kg
	15	4	11	Mare	Palomino	Native X	<400kg
	16	16	10	Gelding	Bay	Shetland	<400kg
	17	17	11	Mare	Bay	Native X	<400kg
	18	16	11	Gelding	Bay	Native X	<400kg
	19	12	10	Mare	Bay	Native X	<400kg
	20	21	14	Mare	Mixed	Native X	>400kg
	21	7	15	Mare	Bay	Thoroughbred X	>400kg
	22	6	13	Gelding	Skewbald	Welsh Cob X	<400kg
Hainford	23	17	15	Gelding	Chestnut	Thoroughbred X	>400kg
	24	25	13	Gelding	Chestnut	Welsh X	<400kg
	25	20	12.3	Mare	Bay Roan	Welsh Mountain	<400kg
	26	18	17	Mare	Black	Shire	>400kg
	27	17	12	Mare	Chestnut	Native X	<400kg
	28	9	12	Gelding	Grey	Welsh Section B	<400kg
	29	16	9	Gelding	Grey/Roan	Shetland	<400kg
	30	13	12	Mare	Bay	Native X	<400kg
	31	25	11	Mare	Bay	Welsh Section A	<400kg
	32	17	12	Mare	Bay	Welsh Mountain	<400kg
	33	19	13	Mare	Grey	Native X	<400kg

Table 8. Demographic details of the horses selected for the field trial

Horse weights are categorised into under or over 400kg to determine supplement dosage as directed by the CP manufacturer. With regard to breed, X refers to a cross-breed, i.e. native X = a native pony breed crossed with an undefined breed.

Field Trial Design

The study was designed as a blinded, three-way crossover, placebo-controlled trial lasting from January to June 2009. Assessment date temperatures ranged from -4°C to 22°C. Horses were located on three farms that were within a 15-mile radius and run under the same management regimen, with all horses permanently at pasture. The three-way design allowed each horse on each farm to receive each of the three substances. However, close to the planned start of the study, the managers of the farms changed the design for a number of reasons which resulted in the horses being split into three groups (n=11) as determined by the farm on which they resided, with one substance per farm. Therefore, the trial became a two-way cross over, with the horses grouped by farm. The trial was then run as follows: all three groups underwent two non-supplemented weeks of pre-trial assessments to ascertain their basal level of stiffness before the trial began. After this period one group (PL) was fed the placebo for the 16-week trial duration, to assess change in stiffness as temperatures increased from winter into spring. The remaining two groups were either fed CP or PT for six weeks then, after a four-week washout period, crossed over onto the other supplement for the remaining six weeks of the trial (figure 51). This cross-over was done to ensure that the horses in two different groups received both treatments, and to examine whether they produced similar results despite differences in location and the order in which they received the treatments.

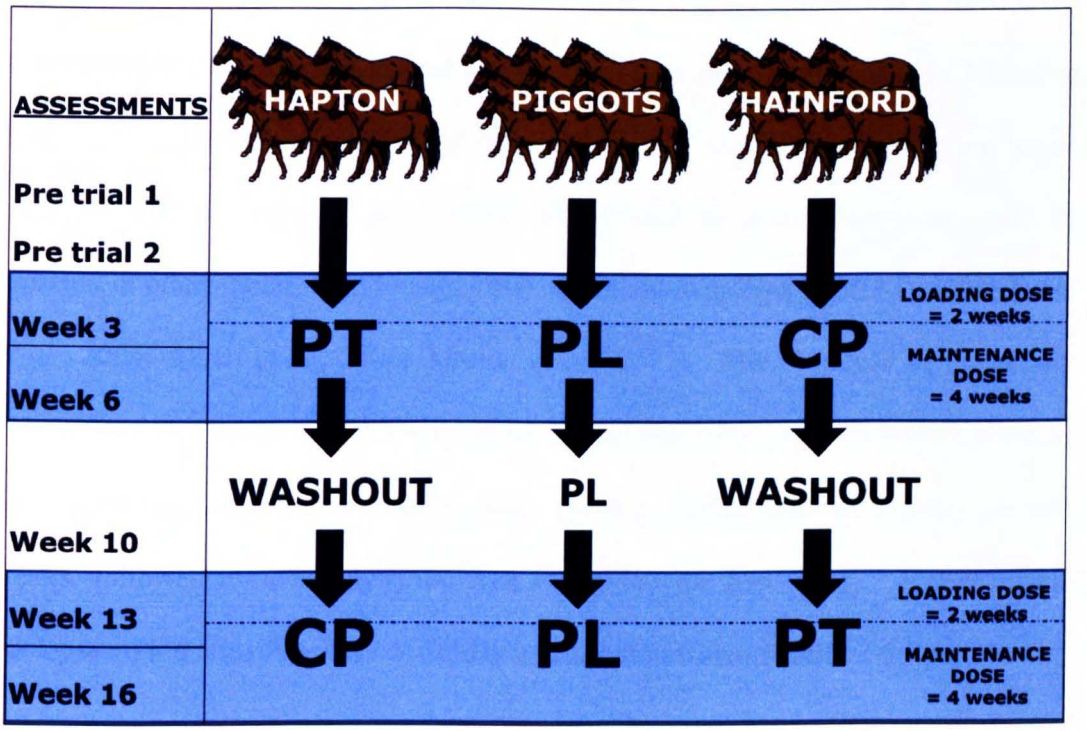

Figure 51. Field trial study design

Three groups of 11 horses were grouped by farm name (Hapton, Piggots, Hainford). One group (Piggots) were fed a placebo supplement (PL) throughout the trial. The remaining two groups were either fed the plant extract cocktail (PT) followed by the commercial product (CP) after a 4-week washout (Hapton), or vice versa (Hainford). All supplements were fed an equal loading dose for 2 weeks, followed by a 4-week maintenance dose.

Supplements were fed in a concentrated bolus of molasses once a day. Dosage of all treatments was determined by the recommended CP dose, which was a fixed dose. MARS Horsecare UK Ltd arranged that the PT (at the same weight as the CP loading dose) contained concentrations of potential active ingredients based on levels recommended for average daily intake, safety and/ or anti-inflammatory activity reported in other species (Tsai *et al.*, 1999; Chainani-Wu, 2003; JECFA, 2004; Secor *et al.*, 2005; Aktan *et al.*, 2006; Altinier *et al.*, 2007). The PL could be fed at any dose. Therefore based upon the CP dosage guidelines, all supplements were given as follows: horses weighing over 400kg were given a loading dose of 14g/day for two weeks, followed by a maintenance dose of 7g/day for four weeks. Horses under 400kg were fed a loading dose of 7g/day and a maintenance dose of 3.5g/day.

Each horse was assessed a total of seven times: at two time points before the trial began, midway (three weeks) through trial periods 1 and 2, at the end of the trial periods (six weeks) and at the end of the four-week washout period. Groups were assessed on three consecutive days to ensure that assessments occurred at a similar time of day. Each group began the study on the day their assessments occurred, so that they had equal time on the treatment. Farrier visits were timed to ensure that horses were not trimmed in the week prior to the assessments.

Assessments were judged by a veterinarian blinded to the trial with previous experience of scoring hindlimb stiffness. All assessments were carried out on hard, level surfaces on each farm. Horses were assessed in walk and trot in a straight line from the front, behind and on either side; and on a tight circle (figure 52).

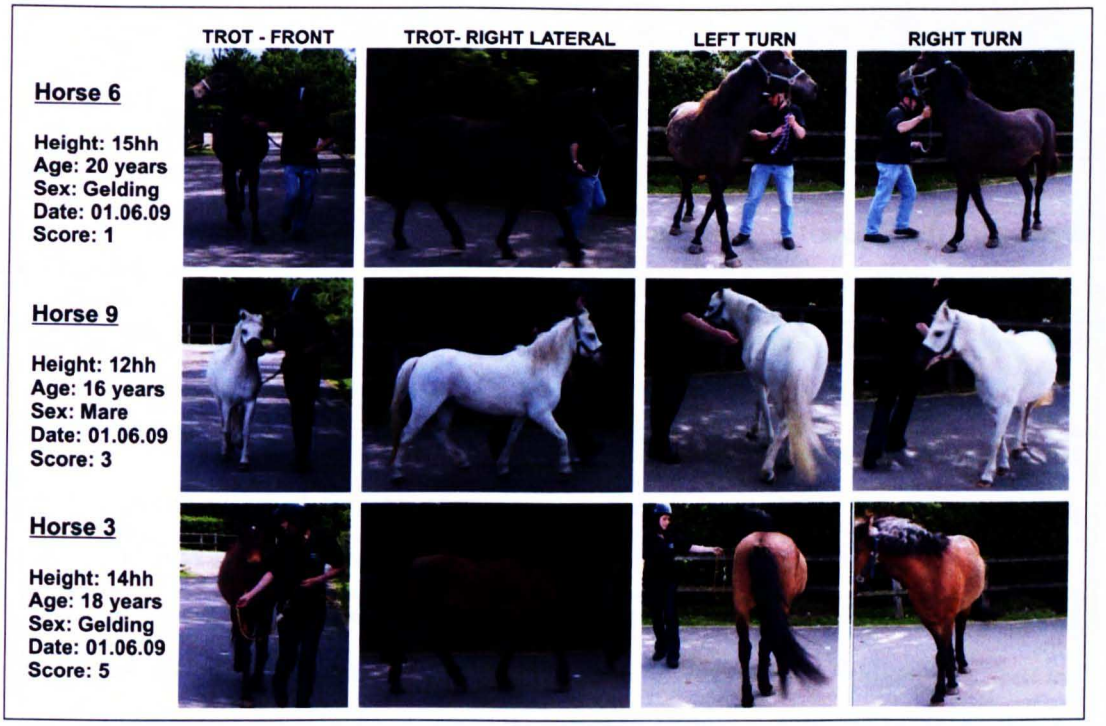

Figure 52. Still video images of three horses ranging from 1 (not stiff) to 5 (very stiff) in score during assessment at Hapton

Horses were assessed in walk and trot from in front, from behind, on both sides and in a tight circle on both reins.

Scoring criteria for the assessments were developed with the veterinary assessor (table 8).

Score	Description
0	Free moving at walk, trot, during sharp turns and on transition from walk to trot.
1	Slightly reduced hock flexion at walk, mainly or only apparent upon tight turns. Moves easily from walk into trot. Trots freely throughout.
2	Mildly reduced hock flexion at walk. Mild stiffness on sharp turns at walk. Some reduced flexion seen on transition from walk to trot. Once in trot, moves freely and shows good flexion.
3	Moderately reduced flexion at walk. Moderate stiffness on sharp turns at walk but demonstrates correct crossover of hindlimbs. Reduced flexion on transition from walk to trot. Once in trot, shows considerable improvement in degree of flexion.
4	Very limited flexion at walk. Profound stiffness on sharp turns at walk, preferring to keep hocks straight with limited crossover of limbs. Slight reluctance to move from walk to trot at transition. Once in trot, slight improvement in degree of flexion. Apparent reduced stride length.
5	No hock flexion at walk. Obvious difficulty on tight turns at walk. No crossover of limbs, preferring to 'pivot' behind. Reluctant to move from walk to trot. Noticeably reduced stride length with short, choppy action. In forward flight, horse lands 'flat footed' or shows 'toe drag' due to lack of hock flexion.

Table 9. Scoring criteria for assessing equine hindlimb stiffness

Statistical Analysis

Intention-to-treat analysis was conducted on the data, meaning that data from all horses that recieved at least one dose of supplement were analysed, including those from horses that did not complete the trial. Only two horses were withdrawn from the trial for medical conditions (unrelated to the trial) requiring NSAID therapy. One horse (Horse 4) was withdrawn before treatment began, and thus was excluded from the whole trial. The other (Horse 8) was withdrawn from the trial after period 1 so only these data were included.

The average of the two-week pre-trial scores and the end of the four-week washout scores were taken as baseline measurements for the succeeding treatment scores in periods 1 and 2 respectively. This was done in case the products were effective and the washout period was too short which may have caused a significant difference between the starting scores and the washout scores. This would have meant that horses were not starting the second phase of the trial on the same stiffness score with which they began the first phase thus meaning that the scores from the treatment periods could not be compared directly. By averaging the two pre-trial scores and using the post washout score, a baseline with which to compare the treatments in both periods could be used.

Change from baseline at weeks three and six were then analysed using a General Linear Model analysis, with horse (nested in treatment), treatment (PL, CP or PT), week (3 or 6) and the interaction between treatment and week as factors investigated in the model. Analyses were performed by a WALTHAM associate, Alison Colyer, using Statistica v8 statistical software and inferences were made using the 5% significance level. The statistical analysis of the *in vivo* data as described relies on the commonly made assumption that there is a consistent meaningful difference

across the measurement scale (e.g. a difference going from 1-2 is the same as going from 4-5). For completeness, a non-parametric Wilcoxon matched pair test (that does not make the same assumption) was also applied to the data. Regardless of which way the data were analysed, the resulting conclusions were the same.

6.3. Results

The results from the trial are shown in table 9.

Location	Horse ID	1st pre-trial	2nd pre-trial	Week 3	Week 6	Week 10	Week 13	Week 16
Hapton	**1**	4	4	4	4	4	4	4
	2	5	4	3	3	4	5	4
	3	4	4	3	3	3	4	5
	4	3	4	X	X	X	X	X
	5	2	3	2	3	3	3	3
	6	2	2	1	3	2	3	1
	7	4	3	5	5	2	3	4
	8	4	3	3	3	X	X	X
	9	3	4	4	4	3	3	3
	10	4	4	4	4	4	3	4
	11	3	3	3	3	3	3	3
Piggots	**12**	3	3	4	4	4	4	4
	13	2	2	2	2	2	2	2
	14	3	3	3	3	2	2	3
	15	2	2	3	2	3	3	2
	16	5	5	5	4	4	5	4
	17	4	3	3	4	5	4	3
	18	3	3	3	3	3	2	2
	19	3	3	4	3	4	4	3
	20	3	4	3	3	3	3	3
	21	2	2	2	3	3	3	3
	22	4	4	3	3	4	3	3
Hainford	**23**	2	3	3	4	3	3	3
	24	2	3	3	3	3	X	3
	25	X	3	4	3	3	3	4
	26	X	2	3	3	3	2	3
	27	3	3	3	3	4	4	3
	28	3	4	4	3	3	4	3
	29	4	5	5	5	5	5	5
	30	3	4	4	4	4	3	4
	31	3	3	4	4	3	3	4
	32	4	5	4	3	4	4	3
	33	3	3	3	3	4	3	4

Table 10. Field trial stiffness score results

Values represent stiffness score (1 = not stiff, 5 = very stiff), **X** *= missing value. Assessments were done twice before the trial began (1st pre-trial and 2nd pre-trial), midway and at the end of the first period (week 3 and 6), after the washout period (week 10) and both midway and at the end of the second period (week 13 and 16).*

All horses started with a stiffness score of between 2 and 5. The median starting stiffness score was 4 for the Hapton group and 3 for Piggots and Hainford groups.

Final group statistics (combining Hapton and Hainford horses as they received both treatments) are listed in table 10. Horse 4 has been excluded from the data as she was removed from the entire trial. Horse 8 underwent the first period after which he was withdrawn from the trial, therefore his data is included in the table.

	Hapton + Hainford	Piggots
Group number	21	11
Mean age	17	14
Youngest	9	4
Oldest	25	23
Median initial stiffness	3	3
Lowest initial score	2	2
Highest initial score	5	5
Weight <400kg : >400kg	14 : 7	7 : 4
Ponies : horses (≤14.2hh : ≥14.3hh)	18 : 3	8 : 3
Median height (hh)	13	13
Average height (hh)	13	13
Smallest pony (hh)	9	10
Highest horse (hh)	17	16
Mares : geldings	10 : 11	6 : 5

Table 11. Vital statistics of horses in the treatment groups (Hapton and Hainford) and placebo group (Piggots)

Initial analyses found the interaction between treatment and week and the main effect of week to be non-significant (p=0.220 and 0.921 respectively), see table 11.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Name(Treatment)	38.88	49	0.794	2.275	0.001
Treatment	6.00	2	3.001	8.605	<0.001
Week	0.00	1	0.003	0.010	0.921
Week*Treatment	1.08	2	0.540	1.549	0.220
Residual	24.41	70	0.349		
Total (corrected)	70.37	124			

Table 12. ANOVA results of trial change from baseline data

Therefore, the interaction and the effect of week were dropped from the model. There were significant differences between treatments, see table 12.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Name(Treatment)	38.89	49	0.79	2.27	<0.001
Treatment	5.94	2	2.97	8.50	<0.001
Residual	25.50	73	0.35		
Total (corrected)	70.37	124			

Table 13. ANOVA results of trial change from baseline data, with interaction and week removed, showing overall effect of treatment

There was variation in hindlimb stiffness score as observed in the placebo group but the average change in stiffness from baseline was not significant with a 95% confidence interval (-0.32, 0.04) (figure 53). There was no significant difference between PT and PL (p=0.763). However, the average change in stiffness from baseline in CP was significantly increased, i.e. horses became more stiff, when compared to both PL and PT (p<0.001 and p=0.001 respectively).

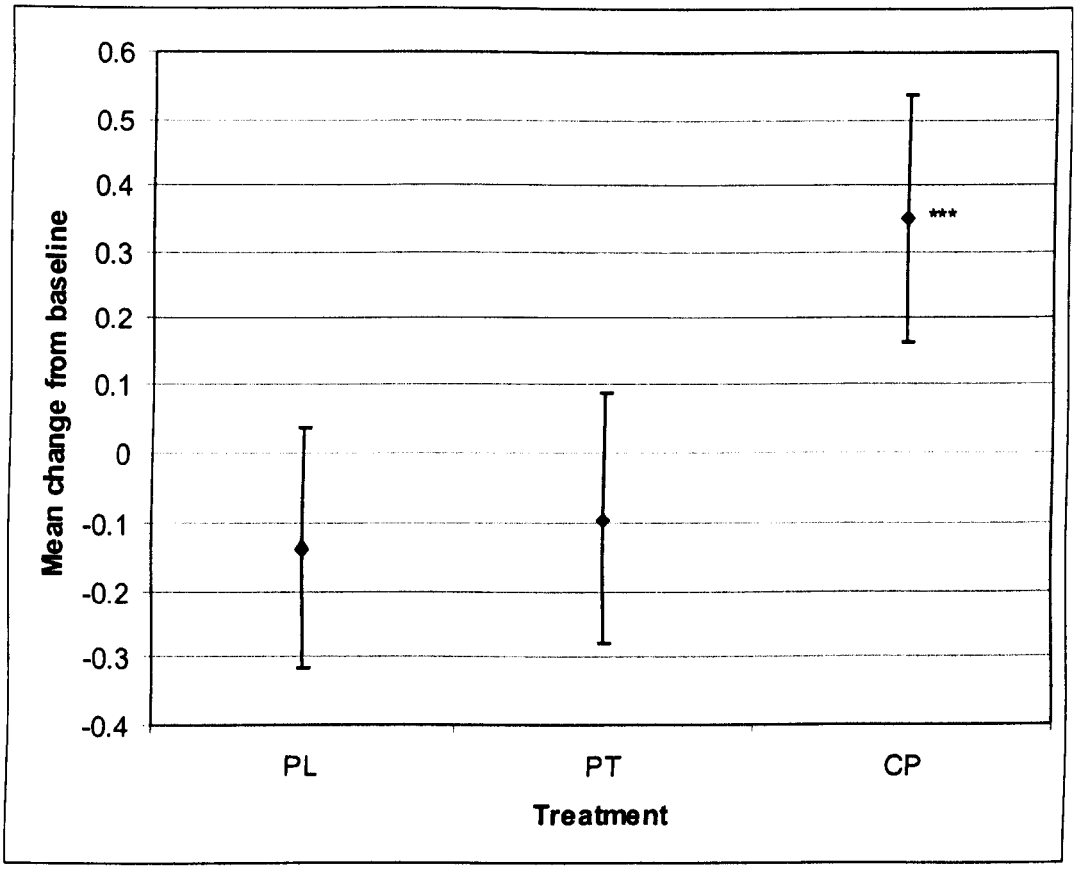

Figure 53. Mean change from baseline stiffness score, with 95% confidence intervals, over 6 weeks in the commercial product (CP), plant extract cocktail (PT) and placebo (PL) groups

*Positive scores indicate increased stiffness, negative scores show improved mobility. Significance compared to PL and PT is indicated by *** ($p<=0.001$). Bars show 95% confidence intervals.*

6.4. Discussion

The aim of the field trial was to determine whether the feeding the PT would result in a detectable clinical improvement in mobility in a group of chronically stiff horses as this is how a typical horse owner would assess efficacy of a supplement. In addition, the method had to be non-invasive to comply with the wishes of the sanctuary. A visibly detectable improvement in stiffness may have been supported by invasive, physiological measures of GAG and inflammation in the blood or synovial fluid. For example, inflammatory mediators, such as cytokines and peripheral blood mononuclear cells (PBMC) increase the release of GAGs *in vitro* (Mastbergen *et al.*, 2002), and levels of GAG in the synovial fluid are elevated in horses with OA (Alwan *et al.*, 1991) and horses with moderate to severe joint disease and lameness (standard grade 1-5) (Palmer *et al.*, 1995). However, the aim was to detect an improvement as would be assessed by an owner. Thus, if the supplement produced a visibly detectable improvement in stiffness but did not alter GAG and PGE_2 levels, then the desired goal would still have been achieved. Consequently, a non-invasive determination of hindlimb stiffness in a group of horses with joint mobility issues was chosen as the outcome measure of *in vivo* efficacy, designed with a prior estimation of *n* numbers for statistical power (Dyson *et al.*, 2001).

Neither the commercial product nor the formulated cocktail of plant extracts significantly improved stiffness in horses under these trial conditions. However, despite considerable individual variation between horses as indicated by the placebo group, a significant increase in stiffness occurred in the CP group compared to placebo and PT groups. Whether this statistical significance reflects a biological significance is questionable as it amounted to an average increase of 0.35 in stiffness score from baseline. However, when looking at the number of horses out of the total

number from both trial periods combined, 35% of horses on CP became worse between weeks one and three (i.e. at the end of the loading dose) compared to 10% on PT and 18% on PL. By week six, this had changed to 25% for CP, 19% for PT and 18% for PL compared to their starting score for the trial period. This trend was replicated in the CP groups in both periods. Interestingly 35% of horses improved between weeks one and three on the PT, compared to 10% on the CP, and yet after six weeks on PT, only 19% were less stiff than they were at the start of the trial period, with 15% less stiff on CP. This initial improvement after the first three weeks on the loading dose suggests that future trials should consider increasing the dosage of PT as the dosage used was based on the CP manufacturer's recommendation. Although, it should also be mentioned that 23% of horses improved on the placebo between weeks one and three, and after six weeks 36% were less stiff than they were at the start of the trial period, thereby highlighting the variability within the horse population studied.

The horses were kept under the same management regimen, in terms of being permanently out on pasture, fed their supplement at the same time of day and only brought in for the farrier or their assessments. This removed the confounding factor of owner variation.

Other joint supplement studies have assessed efficacy in ridden horses (Clayton *et al.*, 2002). However, different intensities and frequencies of work between owners can be a confounding variable in lameness/ stiffness studies. This study used horses that were not exercised and so was able to assess the effect on natural (i.e. not exercise induced) stiffness of horses at pasture.

Stiffness was assessed, rather than assessing lameness which has been done in previous studies (Hanson *et al.*, 1997). Lameness can be multifactorial in

pathogenesis, and is often asymmetric in clinical presentation. By assessing stiffness, one can consider the freedom of movement regardless of lameness. Some studies have used symmetry as a defining variable of improvement (Clayton *et al.*, 2002). However, horses with asymmetrical lameness may become more symmetrical due to becoming lame on the weight bearing limb, giving a false positive result. In addition, false negatives could be introduced by the presence of undetected conditions such as hoof abscesses causing lameness, which would not be alleviated by a joint supplement. Thus by setting the clearly defined criteria for hindlimb stiffness in this study, lameness was not a confounding factor. However, it is recognised that the use of a non-validated, non-standardised scoring system is a serious limitation to this study.

This study used a single subjective method of assessment. Studies using either multiple observers for a subjective assessment (Goodrich *et al.*, 2002), objective methods such as vertical ground reaction forces (Clayton *et al.*, 2002) and gait characterisation software (Forsyth *et al.*, 2006) are possible. In addition, physiological measures, such as quantifying potential biomarkers of inflammation in the synovium (Pearson *et al.*, 2009) can be used alongside subjective assessments to strengthen the conclusions drawn from the data. However, these were not feasible options for this study due to ethical, time and location restraints. Consequently, a visual assessment of stiffness in two gaits was used. The aim was to represent the real life situation to determine whether the supplements caused a detectable change without bias. Bias can be introduced by owners who perceive a false benefit due to the 'placebo effect', thus an impartial veterinarian was used to assess the horses under blinded trial conditions.

The use of only one assessor and the stiffness grading criteria could be criticised in this study. However, numerical scoring systems are considered more reliable when evaluated by a single assessor rather than multiple ones (Fuller *et al.*, 2006). The assessor in this study was an equine veterinarian, experienced in assessing lameness and stiffness, and who defined the scoring criteria based on her experience. Thus, although it was not an established scoring system and may not have been repeatable between assessors, the assessor in this trial did not have to overcome the difficulties of learning and applying a novel scoring system. Despite this, it is acknowledged that experience does not necessarily improve accuracy. A canine study comparing an objective lameness measure (force platform gait analysis) with subjective lameness scores by experienced veterinary surgeons and novice veterinary students found that although the experienced surgeons showed better repeatability in lameness scoring than the novice students between viewings, all observers had poor correlation with the gait analysis data (Waxman *et al.*, 2008). Therefore, although subjective measures with which to compare the scoring system data were not available for the trial, future analysis of the assessment videos by multiple observers, multiple times, would give an indication of inter- and intra-observer reliability.

Clinical trials lacking a blinded assessor introduce conscious and/ or unconscious bias into both attaining and interpreting the results (Bridgman *et al.*, 2003). This trial successfully ensured that none of the handlers, farm managers or the assessor knew which treatments the horses were receiving. In addition, the assessor was a veterinarian impartial to the outcome of the trial. The efforts taken to ensure thorough blinding reduces any potential bias in the results.

Despite efforts to ensure the trial was well-designed, there were limitations, such as the placebo group being on one farm. This was requested by the sanctuary to prevent

confusion with multiple treatments being administered on each farm and to avoid needing to lengthen the trial, which would have interfered with the horses moving to summer pastures. The placebo group was only used to monitor changes in stiffness in that group of horses over the trial duration and it meant every horse on the trial was fed a supplement, thus increasing the blinding of both assessor and staff. The placebo group showed that there was variation in hindlimb stiffness score but the average change in stiffness from baseline was not significant, indicating that the increase in ambient temperature as the horses moved from winter into late spring did not have an effect.

Ideally the study design would have included a positive control group receiving a NSAID to reduce stiffness. This group would have indicated whether the horses were capable of showing a significant improvement in stiffness and, more importantly, whether this was detectable by the assessment method. However, this was not a viable option for ethical reasons and the number of animals that would be required. In addition, it would have meant increasing the numbers of horses which was not possible, as well as increasing the length of the trial which would have interfered with them moving onto new pastures.

As previously mentioned, the trial duration (six weeks on each supplement) was determined by the sanctuary's policy of moving the horses off the farms onto their summer pastures. Whilst a NSAID may have shown an improvement in this time-frame, the duration may have been too short to see a detectable reduction in stiffness by feeding a cocktail of plants extracts. However, it was relevant to the 'in the field' situation where an owner would want to see a benefit relatively quickly. It may be that prospective studies of this nature need to be longer, in terms of years rather than months, although the potential cost of this may be prohibitive.

All horses were kept under the same management regimen in this field trial, but they were not located on the same farm, which introduced variation such as different assessment areas and days, as well as environmental differences. These differences were unavoidable but every effort was made to minimise their impact such as ensuring horses were kept on similar flat pastures and assessment areas were on level tarmac surfaces. To ensure all horses were assessed at a similar time of day, they were assessed on different days. So that all groups were on the trial for the same duration, they began their trial on the day on which they were to be assessed. The treatment study followed a cross-over design and the same results were found regardless of the farm, indicating that the effect of farm was minimal in this study.

This study fed two joint supplements proven to reduce an indicator of cartilage degradation (GAG release) *in vitro* to horses with joint mobility issues to assess their ability in reducing stiffness. Although *in vitro* GAG release and *in vivo* stiffness are not closely linked, the field trial sought to discover whether a gross reduction in stiffness could be seen in horses fed the PT supplement. Despite convincing anti-degradative efficacy in the laboratory, neither supplement significantly improved stiffness in the horses under the management regimen in this study. However, it must be remembered that the *in vitro* model is a simplified model of early OA, using one cytokine to cause an acute effect. This field trial used horses with chronic stiffness, of unknown disease stage and aetiologies. Therefore, it is unsurprising that the *in vitro* and *in vivo* results do not concur. Nevertheless, it does highlight how careful manufacturers should be when directly transferring results from *in vitro* studies to the live animal without field trial support or clinical proof. There are many intermediate steps needed between the acquisition of meaningful laboratory data and the end point of proof of efficacy *in vivo*.

One such issue is bioavailability. Admittedly, many joint health products may have systemic benefits such as anti-inflammatory and pro-analgesic effects, which may manifest in an overall clinical improvement. However, if bioavailability is low then a larger intake is required to be capable of producing any effect. Bioavailability is often an issue when discussing nutraceuticals but it is difficult to determine when the active ingredient is unknown or when studying cocktails containing multiple actives. In addition, care must be taken when transferring data between species, for example, the bioavailability of glucosamine in the dog is 12% (Adebowale *et al.*, 2002) whereas estimates in the horse are under 6% (Laverty *et al.*, 2005).

Although this study showed that *in vitro* models cannot replace *in vivo* studies, they are still useful to help screen nutraceuticals for *in vivo* testing. They also may aid investigation into possible mechanisms of action of nutraceuticals determined to be efficacious *in vivo*. Thus, *in vitro* models are an important tool in assessing nutraceuticals, when used in conjunction with *in vivo* data.

In conclusion, although the commercial product and the formulated supplement showed significant beneficial effects *in vitro* in the previous chapter, neither was able to significantly improve hindlimb stiffness of horses as evaluated in this study. This study was not without its limitations, the outcome measure of a visibly detectable improvement in stiffness may have not been sensitive enough to detect a biological improvement in reduced inflammation/ degradation. However, it sought to discover whether an improvement in stiffness could be detected by a veterinarian blinded to the trial, thus trying to mimic a real life situation whilst removing the bias of owner subjectivity. The lack of improvement in this study highlights the dangers of assuming *in vitro* benefits will translate to the live animal. Thus, manufacturers of joint health supplements should carry out rigorous well-designed trials to prove that

their product can effectively produce the results that they claim. In addition, veterinarians and horse owners should be more aware of the need to critically appraise the evidence provided by nutraceutical companies.

Although the benefits of PT and CP seen in the *in vitro* model were not reflected in the *in vivo* field trial, it does not necessarily mean that they are ineffective *in vivo*. It may be that the methods employed to detect an improvement *in vivo* were not sensitive enough, or that the results were affected by confounding factors such as pain, dosage and trial length. Moreover, the *in vitro* model mimics early stage OA whereas the horses in the field trial were already stiff, showing signs of more advanced disease progression. However, the field trial was important, not only to experience the difficulties and challenges associated with live animal studies, but also to replicate how a joint supplement would be evaluated by a horse owner. A beneficial effect in the laboratory, without a visible improvement in the live animal would not be of interest to many horse owners.

Qualitative methods for assessing equine mobility, such as lameness or stiffness scoring, are subjective and variable. For example, the designation of mild to moderate lameness, even among experienced equine clinicians, is poor (Keegan *et al.*, 1998). In addition, these scoring systems may lack the sensitivity to detect subtle improvements. Identifying more objective, quantifiable markers of improvement, such as protein levels, could therefore be a valuable clinical tool. Using the *in vitro* explant model could potentially aid the identification of biomarkers *in vivo* to indicate improved joint health. Therefore, adapting the model to identify potential markers of structural change in the cartilage secretome highlights how models may be used to inform, improve and expand the quality of science in field trial work.

CHAPTER 7. GENERAL DISCUSSION

The aim of the work in this thesis was to develop and test *in vitro* models of equine cartilage to investigate the potential application of nutritional targeting to support joint health in equine OA. The studies have shown that the *in vitro* models of equine cartilage can be used to detect cytotoxicity, anti-inflammatory and anti-degradative activities of potential joint supplement ingredients alone and in combination. More importantly, in using the models, this work found that curcumin at low micromolar concentrations (3-12μM) significantly reduced IL-1β-stimulated GAG and PGE_2 with no adverse effects on cell viability in equine monolayers and explant cultures, whereas, these effects were concomitant with cell death at 25-100μM. This low-dose protective effect and high-dose toxic effect suggests that curcumin is hormetic in chondrocytes, as it is in other cells. Hormesis is a term used by toxicologists to refer to this type of biphasic dose response to an agent, whereas biologists define it is as an adaptive response of cells and organisms to a moderate stress (Mattson, 2008). Curcumin has been proposed as a hormetic agent, termed hormetin, as low doses of curcumin (below 1μM) stimulate proteasome activity in cultured human keratinocytes to protect them from ageing, whereas concentrations over 3μM are inhibitory (Ali and Rattan, 2006).

The data presented suggest that curcumin may be of benefit to equine joint health, in terms of reducing IL-1β-stimulated GAG, PGE_2 and MMP-3 release from cultured cartilage explants. This reduction in IL-1β-induced GAG and PGE_2 release was also observed when curcumin was combined in a cocktail of other plant extracts with purported anti-inflammatory activity. However, this was affected by the solvent used to dissolve the cocktail.

The explant model was adapted for rapid and high-thoughput proteomic analysis of the media. This enabled the identification of the ECM components and less abundant proteins in the cartilage secretome upon IL-1β-stimulation in this simplified model of mimicking the events in early OA. Some of these markers may be used to assess the effects of plant extracts in the model. In addition, further refinements to the model may help identify biomarkers *in vivo* that can aid OA detection and prognosis, as well as evaluating therapeutic efficacy of anti-inflammatory drugs and joint supplements.

At the same time as the proteomics work was being conducted, an opportunity to manage a field trial arose. The plant cocktail, previously tested *in vitro*, was fed to a group of horses with chronic hindlimb stiffness under field trial conditions to determine whether it produced a detectable clinical improvement in mobility, as would be deemed beneficial by an owner. The plant extract cocktail did not significantly reduce stiffness in the horses after six weeks of supplementation.

Although the results from the field trial suggested that the cocktail was not effective in terms of visibly improved mobility, it is likely that any improvement would have been masked by confounding factors such as pain, conformation, selected dosage and trial duration. It also emphasised the complexities of field trial based work and highlighted the need for markers to, firstly, select an appropriate population of horses at a similar disease stage for future trials and, secondly, detect improvements *in vivo*. These markers may be potentially found in the secretome of explants *in vivo*. Thus, highlighting the potential value of using the *in vitro* explant model to study the cartilage secretome in order to inform and improve future field trial-based work.

Despite the positive findings of this thesis, there are several limitations to the work described.

7.1. Compound Purity

This work highlighted some of the difficulties and limitations when working with extracts rather than pure chemicals. For example, the curcumin tested in chapter 2 was a commercially available product from Sigma-Aldrich with a reported purity of approximately 70%. It could be argued that only pure curcumin should have been tested to ensure that the effects seen in the models were from curcumin alone. However, the curcumin used for the studies has been used by other researchers to determine its effects on other cell types (Ali and Rattan, 2006; Singh *et al.*, 2009), and so there was a benefit in using it to enable comparisons with the published literature. It should be noted here that many commercially available curcumin preparations, including the preparation used in this thesis, are often a mixture of three curcuminoids; curcumin (also known as diferuloylmethane), demethoxycurcumin and bisdemethoxycurcumin (Yang *et al.*, 2007; Kunnumakkara *et al.*, 2008). Thus, the term 'curcuminoid' may be more appropriate to describe curcumin preparations of this nature (Quitschke, 2008). However, for comparative purposes with published studies that use the term 'curcumin', and in the absence of data determining the fractions of the curcuminoid constituents in the specific preparations used for this thesis, curcumin was used to describe the preparations. In addition, this corresponded with the name of the raw material purchased from Sigma-Aldrich.

The commercially available curcumin was also used in these studies as it was more likely to be contained within a joint supplement than pure curcumin which is expensive. Nevertheless, it would be informative to do a study comparing at least three different sources of curcumin with differing levels of purity and curcuminoid content to determine their effects on cytotoxicity, GAG release and other assays used in the models.

7.2. Choice of Solvent

In aqueous solutions, curcumin is insoluble at both a neutral and acidic pH, and although it dissolves under alkaline conditions, it rapidly degrades (Tonnesen and Karlsen, 1985). Therefore, organic solvents are often used to dissolve curcumin. Based upon the published literature, DMSO was used to dissolve the curcumin in the studies for this thesis (Shakibaei *et al.*, 2005; Quitschke, 2008). However, previous studies have dissolved curcumin in different organic solvents such as ethanol and acetone for use in cell culture (Chan *et al.*, 1998; Syng-Ai *et al.*, 2004). Thus, a study comparing the effects of curcumin in different organic solvents would also be useful.

Choice of solvent was also an issue when testing the plant extract cocktail and was identified as a key variable in affecting the *in vitro* outcome. The quantities of solubilised plant extracts in the final DMSO preparation of the cocktail are unknown. Indeed, some extracts such as the water-soluble bromelain may not have dissolved at all. Therefore, chromatographic profiling to identify and quantify the active or typical constituents in any cocktail tested is an important future direction for this work.

7.3. Storage and Stability

All the prepared stocks of curcumin and the test materials used in chapter 4 were aliquotted and frozen until required. A new aliquot of test material was then defrosted for each study so that no sample underwent multiple freeze/ thaw cycles. This was done to ensure batch-to-batch consistency and to reduce the risk of degradation/ loss of activity that can be associated with repeated freeze thawing.

However, it did mean that the length of time being frozen varied from 24 hours to several months, and it is not known what effects these may have had on the actives. Comparing the effects of curcumin frozen for different lengths of time would be a useful addition to the work in this thesis for comparative purposes between studies. In addition, it would aid the setting of criteria for future studies so that preparations can be discarded after a certain amount of time in the freezer. Although, there is no information on the stability of the combined test materials used in chapter 4, previous studies have investigated the stability of curcumin and found that it rapidly degrades in organic solvents, especially at higher temperatures. For example, after 6 hours at 37°C only 6% of curcumin remained from the original amount solubilised in methanol and PBS (pH 7.4) (Mohanty and Sahoo, 2010). A study comparing DMSO-dissolved curcumin in cell culture medium, found that after nine days at 37°C, the percentage of curcumin had rapidly declined from 100% to less than 20% (Quitschke, 2008). Although the rate of decline varies depending on the choice of solvent and method of solubilisation, these studies both suggest that researchers investigating the effects of curcumin on cells in culture should replace the curcumin-containing media daily. For consistency, the test material methodology in chapter 4 was the same as the curcumin methodology in chapter 2, using a single treatment at the start of the same 5-day culture period. Future studies should investigate the effects of replacing treatments daily to mimic the daily consumption of a dietary supplement.

Despite the solubility of curcumin in organic solutions, it has been reported that curcumin degrades rapidly in organic solvents under exposure to light, with traces of degradation products being detected after only 15 minutes of light exposure (Tonnesen *et al.*, 1986). Although, degradation products were not measured in the

studies in this thesis, the curcumin was only exposed to light during dilution and addition to the wells, which would have taken about 10 minutes. However, it would be beneficial to look at whether curcumin had degraded during this time.

The presence of serum in cell culture medium has been shown to enhance the solubility and stability of DMSO-dissolved curcumin (Wang *et al.*, 1997; Quitschke, 2008). Therefore, when examining the difference in toxicity threshold between the monolayer and explant studies, it may be that as the monolayer cells were cultured with FBS, this may have resulted in them being exposed to a higher concentration of curcumin for a longer period. The proportions of curcuminoids in solution also vary with the method of solubilisation in FBS-containing preparations (Quitschke, 2008). Thus, different curcuminoids may have been present in the FBS and non-FBS media preparations used for the monolayer and explant studies respectively. Future work should look at spectrophotometrically determining the curcumin concentrations in media and using reversed phase chromatography to profile the curcuminoids in solution when doing studies of this nature.

There are a variety of methods for improving the solubility and stability of curcumin including the use of the aforementioned FBS. Other methods that have been reported include forming metallocomplexes of curcumin and zinc (Zebib *et al.*, 2010). However, this would mean adding extra ingredients into the medium, which may be unsuitable for the model or the assays used. For example, the proteases, growth factors, cytokines and their inhibitors present in serum (Childs *et al.*, 1982; McKenzie *et al.*, 1990; Knoell *et al.*, 1998). The relatively new concept of nanoparticulate curcumin may be a useful alternative method for improving the solubility and stability of curcumin. This process uses an emulsifier and a polymer to solubilise curcumin, which is then dried to a lyophilised powder that can be

resuspended in aqueous media where it is protected from hydrolytic degradation (Mohanty and Sahoo, 2010). This would mean curcumin could be tested in an aqueous solution removing the need for an organic solvent.

7.4. Metabolite Testing

This thesis also highlighted that the choice of solvent for *in vitro* testing does not take into account the process of digestion in the *in vivo* situation. Therefore, it could be argued that testing the metabolites of curcumin is more relevant to the *in vitro* situation. Some studies have used simulated digests of test supplements to increase the relevance of the product to the *in vivo* situation (Pearson and Lindinger, 2008). Although this is still not directly transferrable to the live animal and does not account for individual variation, it does attempt to address the digestion issue. However, care must be taken to ensure that the method of digestion is appropriate for the species being studied. Using a simulated digest of the cocktail in the model would be a useful future step, not only for increasing its applicability to the *in vivo* situation, but for comparing the results with those obtained for the DMSO and DMEM-dissolved cocktails.

7.5. The Explant Model

Although the explant model allows the study of cartilage and OA-like changes by using various stimuli under relatively controlled conditions, it does have its limitations. As seen in this thesis, the addition of IL-1β caused a relatively large amount of GAG release from cartilage explants (47.47 ± 1.51%) compared to controls (12.55 ± 0.40%) which is roughly a 35% increase (appendix 10) over 5 days.

Thus, the model caused rapid GAG loss over a very short period of time, unlike in OA, especially post-traumatic OA which can take years to develop and has a long latency period until major structural changes are seen (Lotz, 2010). Thus, the suitability of the explant model for OA studies could be questioned. However, inflammatory mediator production and cartilage GAG depletion occur in equine arthritic joints (Palmer *et al.*, 1995; Bertone *et al.*, 2001) and, as shown by others and in this thesis, these are the two factors that may be modulated by nutritional intervention *in vitro* (Pearson *et al.*, 2007; Mathy-Hartert *et al.*, 2009). Thus, the model mimics aspects of inflammation and structural change that occur in early OA, and does so in a relatively short period of time, which is of considerable benefit in the therapeutic evaluation of plant extracts. Whilst these effects may not be directly extrapolated to the *in vivo* situation, the model may be used to identify suitable substances for further testing as potential joint health supplements.

7.6. Additional Models

The relevance of the chondrocyte model to cytotoxicity could be questioned. Although the chondrocyte is the target cell in the joint, other cells such as gut epithelial cells may experience greater concentrations, especially as oral curcumin has been shown to accumulate in the rat intestine (Ireson *et al.*, 2001). More importantly, curcumin is thought to undergo extensive metabolism in the liver, therefore its effect on the hepatocytes may be more relevant (Wahlstrom and Blennow, 1978). However, this thesis sought to determine whether the concentrations of test products that reduced the degenerative effects of IL-1β were toxic to chondrocytes. Although curcumin concentrations (25μM and over) were cytotoxic to monolayer chondrocytes after 5 days, concentrations as low as 3μM

effectively reduced IL-1β-stimulated GAG and PGE_2 release without any detectable signs of toxicity in monolayer cultures or explants. Therefore, although the safety of the compounds to other cell types or the live animal cannot be determined from these studies, they show that the beneficial effects of low curcumin concentrations observed in the explant model were not due to chondrocyte death.

With regard to culturing conditions, hypoxia and 3-D culture may be more appropriate environmental conditions for chondrocyte culture. The chondrocytes are dispersed through the ECM of the articular cartilage and are exposed to very low O_2 gradients estimated to be 5-7% at the surface to 1% in the deepest layers (Silver, 1975; Zhou *et al.*, 2004). Chondrocytes cultured in monolayers *in vitro* de-differentiate and lose their chondrocyte phenotype (Benya *et al.*, 1978; Grundmann *et al.*, 1980; Evans and Georgescu, 1983; Stokes *et al.*, 2001). However, de-differentiated chondrocytes will re-differentiate back to a chondrocyte phenotype when cultured in alginate beads in low O_2 tensions (5%) (Murphy and Sambanis, 2001; Domm *et al.*, 2002). Culturing chondrocytes in low O_2 also increases cartilage anabolism and reduces production of catabolic enzymes; MMP-1 and MMP-13 (Strobel *et al.*, 2010). These effects are mediated by the hypoxic-inducible alpha subunit of the hypoxia-inducible factor 1 (HIF-1) transcription factor, known as HIF-1α (Strobel *et al.*, 2010). HIF-1α has also been shown to be a critical factor for chondrocyte survival (Schipani *et al.*, 2001). Thus, future studies examining the effects of plant extracts on isolated chondrocytes should consider using 3-D cultures in low O_2 tensions relevant to the joint to increase the physiological relevance when studying isolated cells. Low O_2 tensions may also be useful for studying cartilage explants. Steady rates of proteoglycan synthesis have been reported in bovine explants cultured in 6% O_2, which were comparable to those in fresh cartilage, and

differed from the increased synthesis in explants at 24% O_2 (Ysart and Mason, 1994). All chondrocyte and explants cultures used for the studies in this thesis were incubated in O_2 tensions around 17% in the primary cell culture incubator. Future studies should consider the application of more physiologically relevant O_2 tensions to the models.

Culture duration and passage number have also been shown to affect the chondrocyte phenotype. Articular chondrocytes grown in monolayers have been shown to de-differentiate into fibroblast-like cells, significantly reducing collagen type II expression and synthesis after one to two passages (Benya *et al.*, 1978; Schnabel *et al.*, 2002). The studies in this thesis used first, second and third passage chondrocytes, therefore, in addition to the fact the monolayers were incubated in normoxic conditions, it must be considered that the chondrocytes may have de-differentiated. Some markers of chondrocyte de-differentiation include loss of collagen type II synthesis, a change in the collagen type I: collagen type II ratio in favour of collagen type I (Marlovits *et al.*, 2004), and loss of COMP and collagen type IX expression (Zaucke *et al.*, 2001). Future work could include examining expression of these markers in order to determine whether the cells are still expressing the chondrocyte phenotype.

Researchers in the OA and cartilage biology field are also using co-culture models. Considering OA involves all the joint structures, co-culturing with synoviocytes, the cells next to the external surface of the cartilage, or synovial explants could be more relevant to the *in vivo* milieu and could produce different results. Co-culturing cartilage explants with synoviocytes has been shown to reduce the degradative effects of IL-1β compared to culturing cartilage alone, thus suggesting a protective role of the synovium (Gregg *et al.*, 2006). Synoviocytes were isolated and cultured

during the course of this thesis, but were not characterised or validated with markers, and so these data have not been included. However, the co-culture model is a relevant model of the joint, and these models are of increasing interest as researchers attempt to address the multi-structural nature of OA.

The studies in this thesis used a single addition of the test material concurrently with the addition of IL-1β. Previous studies using curcumin have pre-treated cartilage explants with IL-1β for a period of time before adding the test material, to replicate the situation of using the supplement once the cartilage is diseased (Schulze-Tanzil *et al.*, 2004; Shakibaei *et al.*, 2005). However, joint supplements are often used prophylactically. In addition, the purpose of the studies was to use nutritional supplementation to improve joint health. Therefore, it could be argued that it would be more relevant to pre-treat cartilage with the test substance before adding IL-1β. This would determine whether using the supplement would protect the cartilage from an inflammatory insult.

A major limitation of the model was that the explants were not subjected to mechanical load, which is known to stimulate cartilage turnover. There are many published studies that have reported stimulatory effects of cyclic (dynamic) loading on the synthesis of aggregating and non-aggregating proteoglycans by chondrocytes in cartilage explants (Palmoski and Brandt, 1984; Visser *et al.*, 1994) Thus, low-level intermittent loading could help replicate the joint environment. Excessive (injurious) mechanical load on the other hand causes mechanical failure of the tissue, decreases cell viability and stimulates cartilage degradation (Quinn *et al.*, 1998; Loening *et al.*, 2000; Kurz *et al.*, 2001). Degradation is also observed when injurious mechanical load is used in combination with exogenous cytokines (Patwari *et al.*, 2003). Therefore, mechanical loading could be another useful addition to the model

to either help replicate the biomechanical forces in the joint environment, or as another pathophysiological stimulator of cartilage degradation.

7.7. Additional Assays

With regard to the assays, using GAG release alone as an indicator of net GAG release can be misleading. Ideally, additional measurements of GAG synthesis are needed to determine whether any test material can reverse the inhibitory effects of cytokines on proteoglycan synthesis. Previously it has been shown that the inhibitory effect of IL-1β on GAG (namely keratan sulphate) synthesis is mediated, in part, by the stimulation of PGE_2 production (Fukuda *et al.*, 1995). Consequently, when testing curcumin and PT, PGE_2 was measured in conjunction with GAG release. A reduction in PGE_2 in conjunction with GAG release, in the absence of toxicity, was therefore determined as being beneficial. However, it is recognised that future work should address GAG synthesis as well for completeness.

In addition, investigating aggrecanase and metalloprotease activity using enzyme assays or zymograms, rather than confirming the presence of the protein by immunoblotting would provide useful functional information about the model. Furthermore, this work could be supported by identifying the products of aggrecanase and metalloproteinase activity by western blotting with monoclonal antibodies to identify catabolic neoepitopes on proteolytic degradation products, e.g. BC-4 and BC-14 recognise sites of MMP cleavage on aggrecan; BC-3 and BC-13 recognise sites of aggrecanase cleavage on aggrecan (Hughes *et al.*, 1995; Little *et al.*, 1999). This would provide a greater insight into the mechanisms of GAG release in the model and the actions of plant extracts upon them.

Further studies into the mechanistic effects of curcumin and the PT on IL-1β-stimulated cartilage would be useful. It has been shown that curcumin reduces IL-1β-stimulated MMP-3 release from cartilage because of NF-κB inhibition (Schulze-Tanzil *et al.*, 2004). However, it would be interesting to determine the effect of curcumin on receptors to determine whether curcumin is down-regulating the response to IL-1β and/or reducing ability of IL-1β to have an effect. For example, does curcumin down-regulate the surface receptors for IL-1β, or increase production of its decoy receptor, IL-1RII?

Similarly, further studies are needed to determine the impact of significantly reducing IL-1β-stimulated PGE_2 release. For example, although COX-2 inhibiting NSAIDs reduce the pain and inflammation associated with PGE_2 production, they shift the PGE_2:TxA_2 ratio in favour of TxA_2 (Penglis *et al.*, 2000). TxA_2 has been shown to increase IL-1β and TNF-α synthesis by monocytes (Caughey *et al.*, 1997). Therefore, COX-2 inhibition is not always beneficial, especially in the long term. Curcumin has been shown to reduce COX-2 expression in TNF-α and IL-1β-stimulated chondrocytes via inhibiting NF-κB (Shakibaei *et al.*, 2007). Thus, it would be beneficial to determine the effects of curcumin on the synthesis of other prostaglandins and TxA_2. In addition, COX-2 inhibition could reduce PGH_2 synthesis, from which a variety of prostaglandins are synthesised. Therefore, inhibiting PGE_2 release further down the eicosanoid pathway downstream of PGH_2 may be more beneficial, especially via inhibiting cytokine-inducible synthases such as mPGES-1 (Kojima *et al.*, 2004). Further work could examine the effect of curcumin and the PT on the expression and production of mPGES-1 by IL-1β-stimulated chondrocytes and cartilage explants.

With regard to the western blots, no protease inhibitors were added to the samples after collection. Although samples were stored at -80°C, further degradation of ECM components may have occurred post-collection, especially considering the increased levels of MMP-3 in the IL-1β-treated samples. Therefore, future work should consider the use of EDTA and protease inhibitors in the samples before they are stored for analysis.

7.8. Biomarker Validation and Quantification

The use of the *in vitro* explant model for rapid proteomic analysis of the cartilage secretome as documented in chapter 5 may aid biomarker identification. An additional technique, stable isotope labelling, has recently been used to determine *de novo* proteins in the secretome of chondrocyte monolayer and cartilage explant samples (Polacek *et al.*, 2010). This approach could be expanded to determine whether proteins released from IL-1β-treated explants are synthesised and released in response to treatment, or are simply leaching out of the cartilage matrix. A comparative analysis of the secretome from cartilage treated with different stimuli, alone and in combination, would also be useful to determine how each stimulus affects the model. Despite the exciting potential of this system, it must be remembered that potential biomarkers require extensive validation and qualification. Levels in both normal and disease states in a large population need to be evaluated and standards must be set to address individual variation as well as other variables. For example, the widely discussed potential biomarker COMP has considerable diurnal variation and an estimated half life of 7.4 hours (Andersson *et al.*, 2006; Kong *et al.*, 2006). Serum COMP levels in humans are raised in response to exercise such as 30 minute walking (Mundermann *et al.*, 2005). Therefore, sampling times

could significantly affect the results. In addition, COMP is non-cartilage specific, e.g. it is elevated in tendons under high load, compared to lower loads (Smith *et al.*, 1997). This suggest that levels of COMP in the serum and urine may not be specific markers of OA, and may be unreliable. However, the state of the molecule, such as its molecular weight and its post-translational modifications, or its cleavage and fragmentation by proteases may be more important biomarkers of disease rather than the intact molecule. Accordingly, COMP fragments have been shown to increase in the synovial fluid after joint injury and in early OA (Lohmander *et al.*, 1994). Fragments of other matrix associated proteins have been also proposed as biomarkers of OA in the synovial fluid, such as collagen type II C-propeptide fragments (Lohmander *et al.*, 1996) and proteoglycan fragments (Lohmander *et al.*, 1989). Studies of the cartilage secretome will aid the identification of other matrix component modifications and fragments released in response to various degradative stimuli.

In addition, although this model may identify potential markers of cartilage degradation, future work determining their relevance *in vivo* may be difficult due to the method of sample collection interfering with the results. For example, repeated arthrocentesis was shown to significantly increase NO, GAG and PGE_2 levels in the synovial fluid of exercised and non-exercised horses (van den Boom *et al.*, 2005).

Despite these considerations, the model provides a simplified overview of the complex environment of the joint, as well as the multifaceted nature of OA. Thus, it may be a useful tool to reduce the background noise inherent when dealing with clinical samples, thereby allowing the identification of potential indicators of ECM structural changes.

7.9. Field Trial Limitations and Improvements

With regard to the field trial, there were significant but unavoidable limitations with the design and methodology of the trial, which were addressed in chapter 5. However, the trial was invaluable for gaining experience in this area and cultivating new ideas for improving any future trials.

7.9.1. Animal Selection

All horses were selected for the trial on the basis that they were chronically stiff but did not require medication. Horses were selected by the farm managers and so were not randomised, which was a major flaw of the trial. In addition, the population selected consisted mainly of ponies (26 ponies:7 horses). Ponies are lighter than horses and so would experience lower loading forces on their joints. Although OA affects both horses and ponies, and occurs naturally in the joints of wild ponies, it has been suggested that the process is hastened by the stresses of heavy work such as racing (Cantley *et al.*, 1999). Some of the horses in the trial were ex-police horses and ex-racehorses, which would have been more predisposed to joint problems than ponies due to their intensive workload history. However, detailed evaluations of the joints were not conducted, such as X-rays and magnetic resonance imaging, to identify the extent of cartilage damage and possible causes of stiffness, such as bone formation limiting movement. Thus, both horses and ponies were classified as being clinically stiff of unknown aetiology. This meant that some animals may have been more able to respond to the mode of action of the actives than others, which increases the variability of the results. Although well-controlled clinical trials can reduce the variability associated with field trials, they are expensive and less

applicable to the in-the-field situation. Thus, the aim of the trial was to represent how supplements are fed to horses in the field where variability in population and cause of stiffness are commonly encountered.

7.9.2. Animal Grouping

The method of grouping animals by weight (being either under or over 400kg) was selected upon the recommendation of the CP manufacturer. However, weights were taken from the records at Redwings, so they were not necessarily accurate at the time the study began. Future work would ideally weigh, or use a weigh tape, to determine the weight of each animal at the start of the trial. Considering the study ran from winter to spring, the weights of the animals would have changed, thus some that may have been under 400kg at the start may have been over 400kg at the end, yet dosing did not change from the start of the trial. Some horses that gained weight were placed on restricted diets over the trial. However, the grouping of animals by weight is still a problem, as theoretically a 399kg pony would receive half the dose of a 401kg pony. Thus, in the future, it would be best to only study animals that qualify for the one dose, i.e. only ponies less than 400kg or only horses more than 400kg, or to use larger numbers of both groups, each on their set dose, but analyse them separately. Ideally, dosages would be individually calculated as weight/kg of bodyweight.

7.9.3. Future Study Design

Any future trials need to ensure that the treatment groups are spread between farms, to remove the farm effect variable. Ideally, all horses would then receive all three

treatments and all treatment orders (6 groups) on all farms. This would require 18 horses per farm (3 horses per treatment order), totalling 54 horses. The proposed trial design for one farm is shown in table 13. A fourth group receiving a NSAID would ideally be included as a positive control, but this would be unlikely if the study was carried out at the same establishment, and would require more horses and a longer trial duration. The practicalities of finding even 54 horses may be a limitation. Using horses in private homes is an option, but the confounding factor of environment would be large and therefore the study may require larger numbers of horses. In addition, there would be less control in the running of the study.

WEEKS	1 2 3	4 5 6 7	8 9 10 11	12 13 14 15	16 17 18 19	20 21 22 23	24 25 26 27
PHASE	PRE-TRIAL	PHASE 1	WASHOUT	PHASE 2	WASHOUT	PHASE 3	WASHOUT
ASSESSMENT	1 2	3 4	5 6	7 8	9 10	11 12	13 14
TREATMENT ORDER (all farms)		PL		PT		CP	
		PL		CP		PT	
		PT		PL		CP	
		PT		CP		PL	
		CP		PT		PL	
		CP		PL		PT	

Table 14. Field trial re-design for a single farm

Grey columns indicate periods without supplementation, and the phases indicate periods with supplementation of either placebo (PL), plant extract cocktail (PT) and commercial product (CP). The six rows represent each combination of treatments, which would be given to 3 horses per farm.

The required loading dose for 2 weeks followed by maintenance dose means that future trials should assess at 2 and 4 weeks rather than 3 and 6 weeks as before. However, the loading dose for the plant cocktail and placebo in this thesis was dictated by the manufacturer of the commercial product. Therefore, more work on bioavailability would probably be needed to determine the appropriate dose. Despite

this, if circumstances would allow, then a longer trial duration would be preferential as a 6-week trial may be too short to produce a visible improvement. In addition a longer washout period may be desirable to reduce the risk of carried-over effects. However, extending the trial length would have to be reconciled with parameters that are most important to control for, such as the seasonal movement of horse between pastures.

7.9.4. Assessment Method

The method of assessment is another area that requires improvement in any future trials. There are various non-invasive methods available to assess gait, but they all have their limitations. For example, stationary force-plate analysis accurately quantifies ground reaction forces, but is limited to only one stride thereby not accounting for stride to stride variation (Merkens *et al.*, 1988). Force-measuring treadmills allow the continuous measurement of ground reaction forces, but are expensive and require the horses to be trained to the treadmill beforehand (Weishaupt *et al.*, 2002). Force-measuring shoes have been used to collect data on ground reaction load from horses at all gaits on the treadmill (Roland *et al.*, 2005). Treadmills can also be used with video recorders to perform three dimensional gait analysis, which objectively quantifies lameness and stride length (Peloso *et al.*, 1993). However, treadmill work has been shown to affect gait (lengthened stance phase) compared to over ground locomotion (Buchner *et al.*, 1994). Thus, sensor-based motion analysis provides an alternative option as it is not restricted to a treadmill. This method uses sensors located on various parts of the horse to locate lameness and provide continuous data as the horse moves (Keegan *et al.*, 2002). Although this assesses lameness not stiffness, it can quantify symmetry or

asymmetry. However, all these systems are expensive and require docile animals, which make them unlikely options for future trials at the sanctuary. Therefore, future trials will need to address the stiffness assessment, as it is not an established or validated method.

7.9.5. Stiffness Assessment Validation

As previously discussed in chapter 5, lameness assessments were not appropriate for these trials and the restrictions placed upon the trial by those involved, necessitated the development and use of the stiffness scale. In order to validate the scoring system, repeatability studies are needed to assess consistency in allocating stiffness grade and ability to distinguish between grades, by both the assessor used in the trial and other clinicians. Video recordings of each assessment are available for this to be done in the future. The advantage of video recordings is that they can be mixed up in random order, so all assessments of a single horse can be seen (in random order) one after the other, which would assess consistency better than seeing the horse weeks apart. All the horses and assessments could also be mixed at random to assess repeatability. The main disadvantage is that the video was not in set place each time and varied between farms, so the variation in angles at which the horses are viewed may affect the results. For both the video footage and future trials, a global scoring system would be a more appropriate measure of efficacy, and has been shown to be more reliable for a clinical assessment of change in lameness both intra- and inter-assessor (Fuller *et al.*, 2006). The global scoring system would simply state whether the animal improved, declined or stayed the same, rather than categorising degree of stiffness. This may be a more accurate method of assessment as, naturally, there is considerable individual variation in range of motion. Thus, it may be that some of

the horses on the trial were not capable of reaching a score of one due to their conformation or the presence of disease limiting movement, e.g. being straight in the hock or bone formation especially in the hock. Kinetic data would have detected whether each horse had more or less range of motion than before, but this method was not possible due to time, location and financial restraints. In addition, the disease status of each animal was not known in the trial so differing disease stage would have been a confounding factor in the trial. If proven biomarkers for the disease stage are discovered in the future, they could be used to ensure that animals of the same disease stage are selected for field trials to reduce variability.

7.9.6. Improving the Stiffness Scoring System

It may be valuable to regulate the assessment area by drawing a line along which the horses have to walk (same length on each farm) so they are assessed at the same set distance, with a video camera set up at the exact same distance in front and from the side at each assessment. This would enable additional post-assessment analysis using the video recordings. In addition, a measure of stride length could be incorporated into the assessment, such as marking the floor or walking the subjects through a dye or chalk dust. However, factors such as reluctant horses, rain and time may make this difficult. Therefore, it can be seen why a quantitative measure of a physiological parameter, such as a biomarker, would be of benefit in field trials of this nature, where circumstances permit.

7.10. Summary

The work in this thesis is summarised in figure 54.

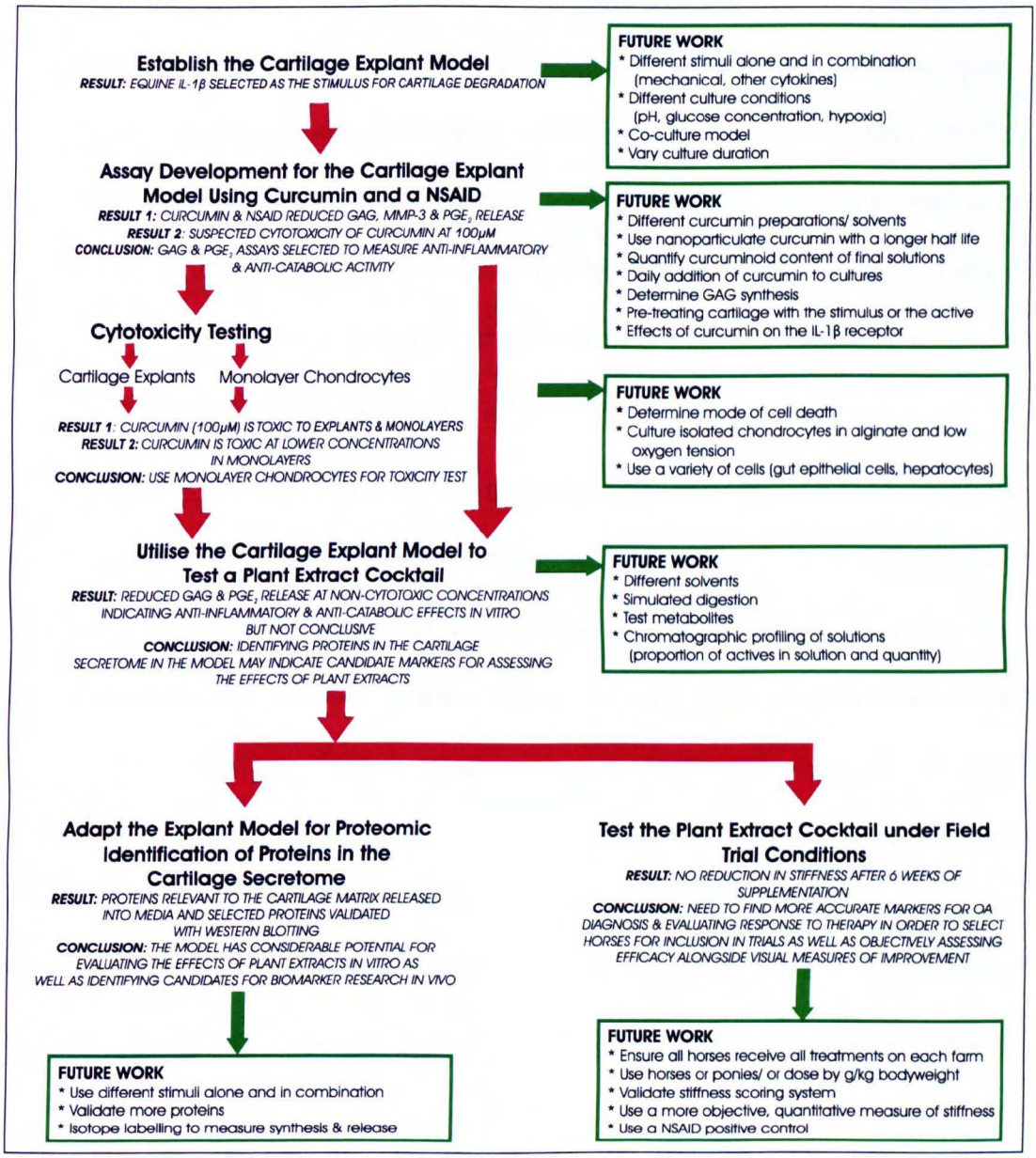

Figure 54. Diagrammatic summary of the thesis and potential areas for future work

Red arrows indicate the flow of the studies in the thesis, green arrows highlight areas for future work. Abbreviations are as follows: glycosaminoglycan (GAG), interleukin-1beta (IL-1β), matrix metalloproteinase-3 (MMP-3), nonsteroidal anti-inflammatory drug (NSAID), prostaglandin E_2 (PGE_2).

In agreement with the proposed aim, the work in this thesis has shown that it is possible to establish *in vitro* models of cartilage degradation and inflammation, consistent with early stage OA, i.e. before collagen degradation occurs in this model. *In vitro* testing of nutritional intervention with plant extracts reduced GAG, MMP-3 and PGE_2 release from IL-1β-stimulated cartilage explants consistent with them having the potential to support joint health. In addition, this model can be used to aid in the screening of novel compounds or help identify potential modes of action by which the ingredients work on chondrocytes *in vivo*.

The equine cartilage explant model was then adapted to identify potential biomarkers in the secretome of IL-1β-stimulated cartilage with or without an anti-inflammatory drug, in comparison to unstimulated controls. This adaptation may aid the understanding of the processes involved in OA as well as the identification of OA markers for diagnosis, disease progression, prognosis and response to joint supplement therapy. The future hope for this work is that it will discover more sensitive markers of improvement that can then be detected *in vivo*. It is unclear whether these markers will translate from *in vitro* to *in vivo*, but it is an important approach for advancing knowledge in this area and improving the scientific quality of future field-based trials.

The modulation of GAG and PGE_2 by plant extracts in the model, suggest that the model may be useful for nutrigenomic studies. Nutrigenomics studies the influence of nutrition on the genome (Müller and Kersten, 2003). The nutrigenomic approach can examine the differential transcriptions (e.g. in signalling pathways) between cells treated with or without nutritional compounds via studying the transcriptome, proteome or metabolome (van Ommen, 2004). Therefore, the anti-inflammatory effects observed in the model in response to plant extracts indicate that it will be a

useful tool for future nutrigenomic studies investigating the effects of plant-derived products on both short-term changes, such as gene expression, and long-term changes, such as protein synthesis, in the cartilage.

The novel findings of this project are summarised as follows:

1. Curcumin reduced IL-1β-stimulated GAG, PGE_2 and MMP-3 release in the equine cartilage explant model at non-cytotoxic concentrations.
2. Curcumin (25μM) significantly increased equine chondrocyte death in monolayer cultures after 5 days, but not in cartilage explants.
3. This project was the first to test the effects of a novel plant extract cocktail on PGE_2 and GAG release from the model. It was also the first to study the effects of the commercial joint health supplement, Cortaflex, in the explant model.
4. High-throughput proteomic technology and quantitative western blotting enabled the first study of the equine cartilage secretome identifying and validating the most abundant proteins present in control, IL-1β and NSAID treated explants.
5. Finally, the results from the field trial were the first to suggest that Cortaflex does not produce a detectable clinical improvement in mobility, in a group of horses with chronic hindlimb stiffness.

In conclusion, this thesis as a whole suggests that there is considerable potential in using *in vitro* models of equine cartilage to assess nutritional intervention to target inflammatory pathways and catabolic mediators to support equine joint health.

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APPENDICES

Appendix 1 Cartilage explant harvesting and model set up diagram

Appendix 2 Glycosaminoglycan (GAG) assay diagram

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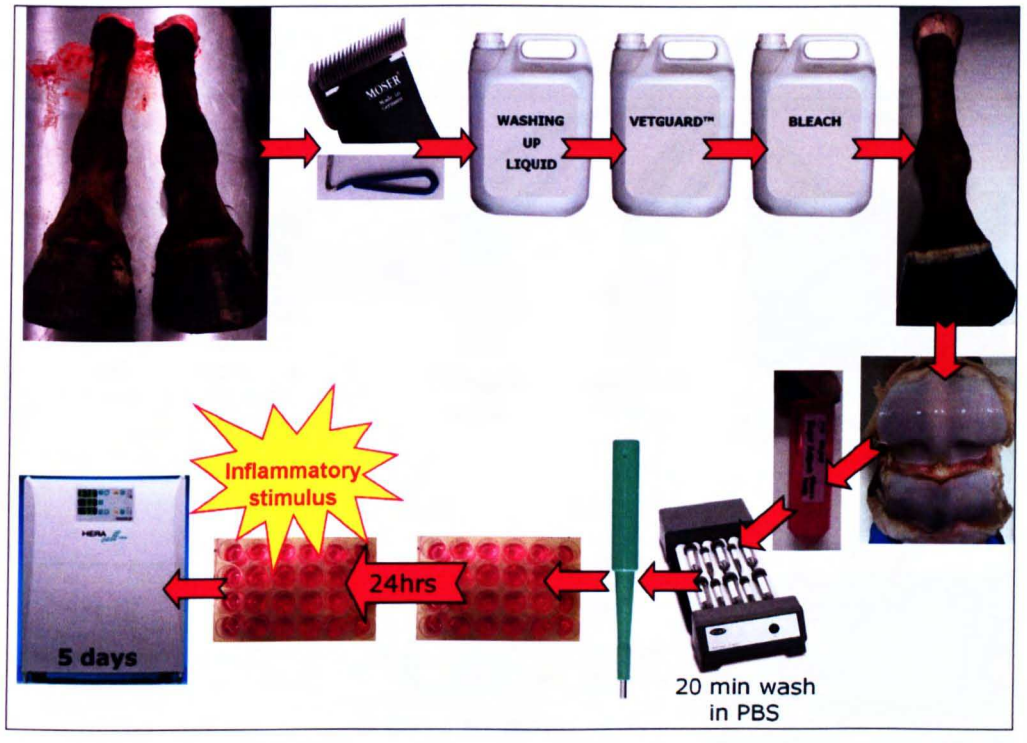
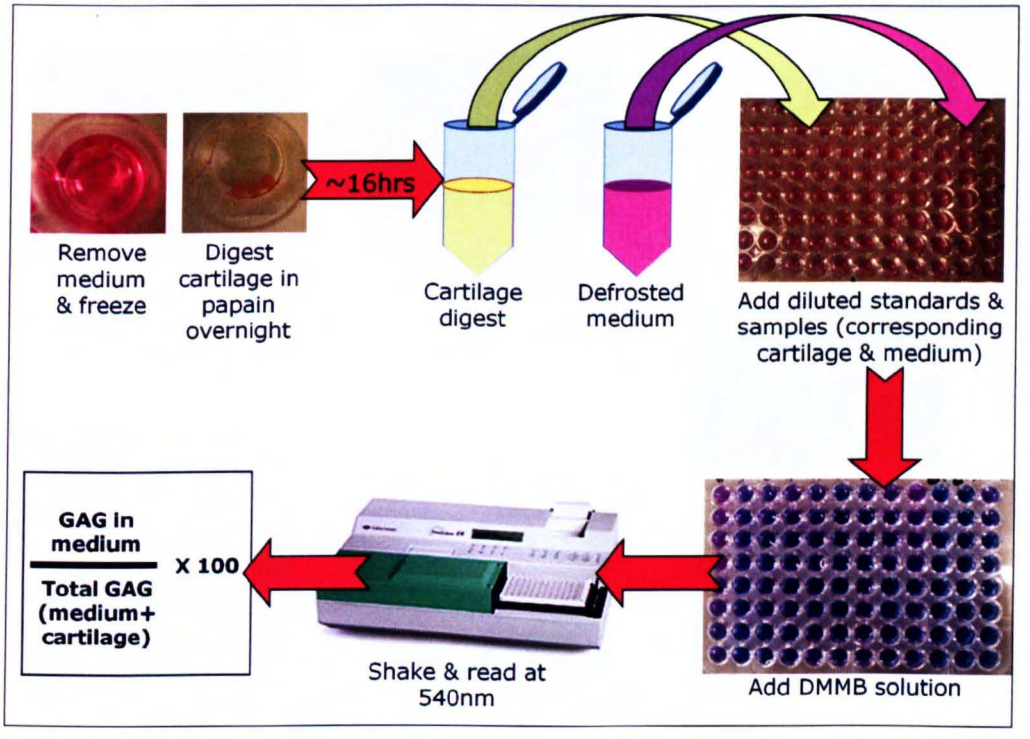
Appendix 1.

Cartilage explant harvesting and model set up diagram



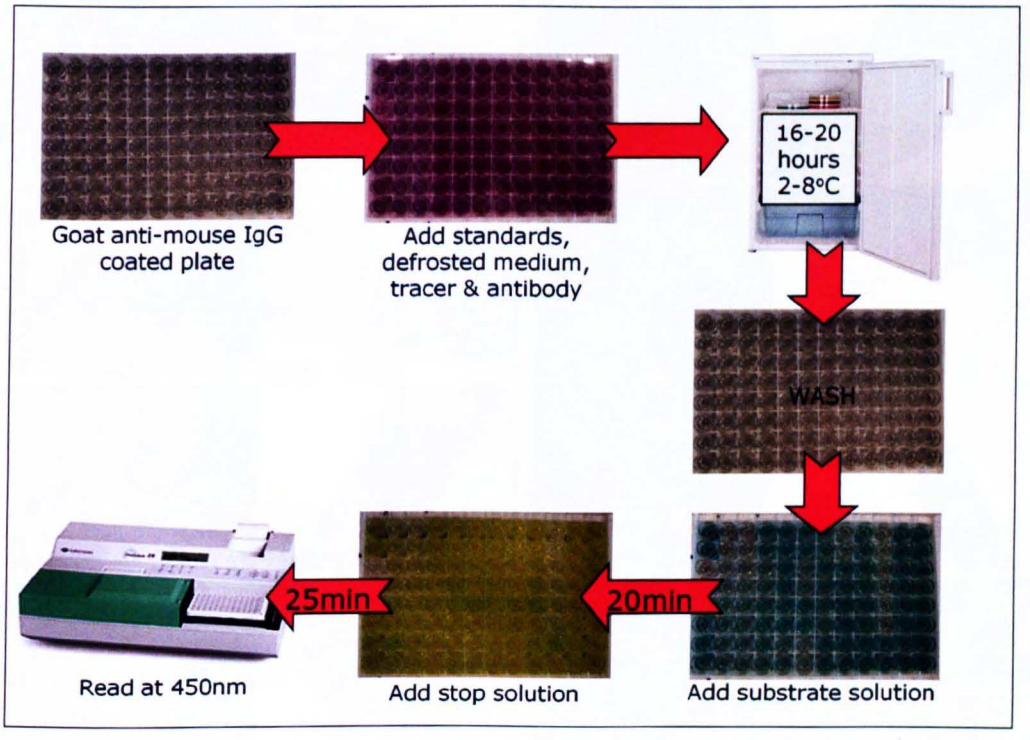
Appendix 2.

Glycosaminoglycan (GAG) assay diagram



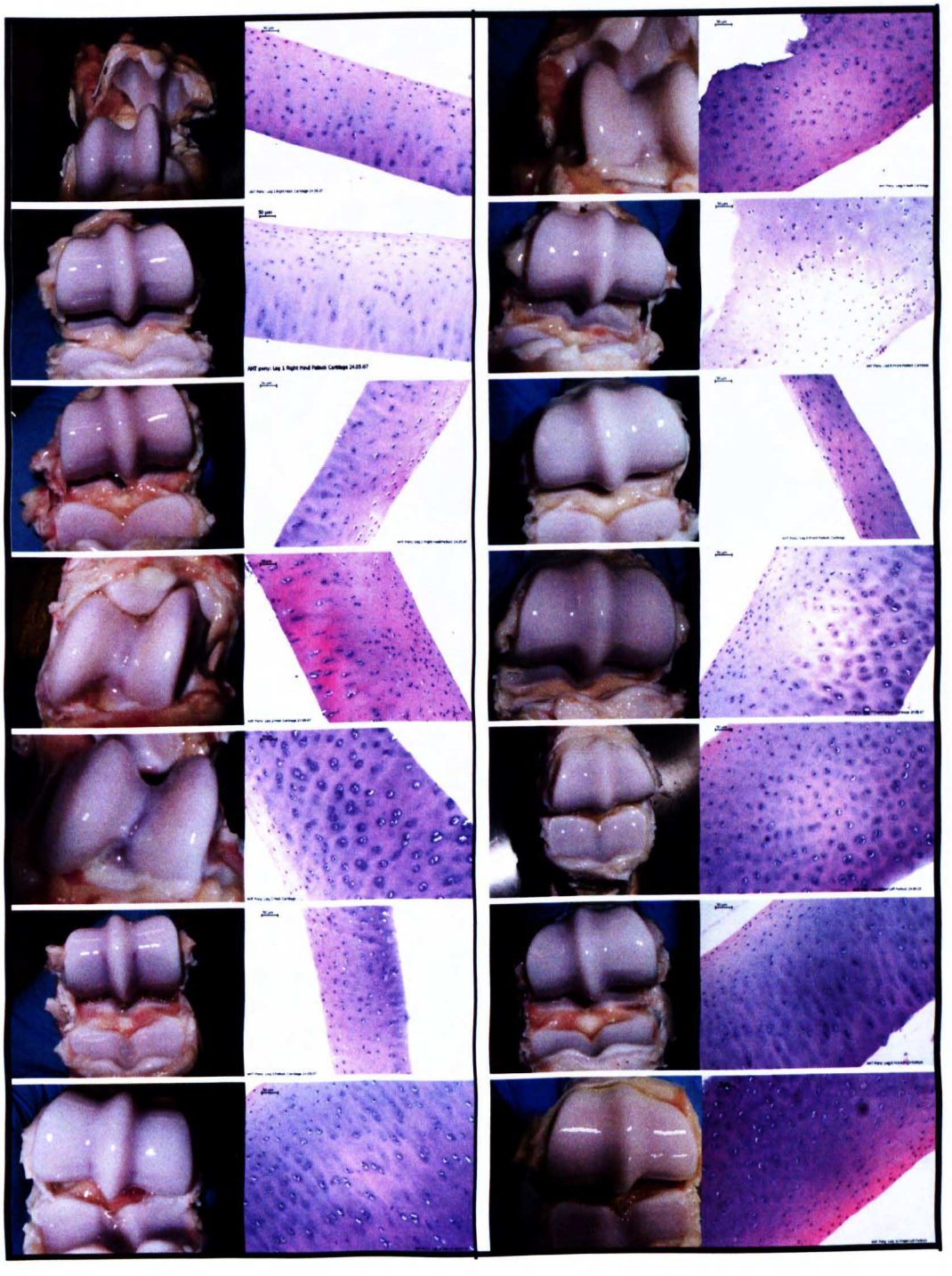
Appendix 3.

Prostaglandin E_2 (PGE_2) assay diagram (high sensitivity method)

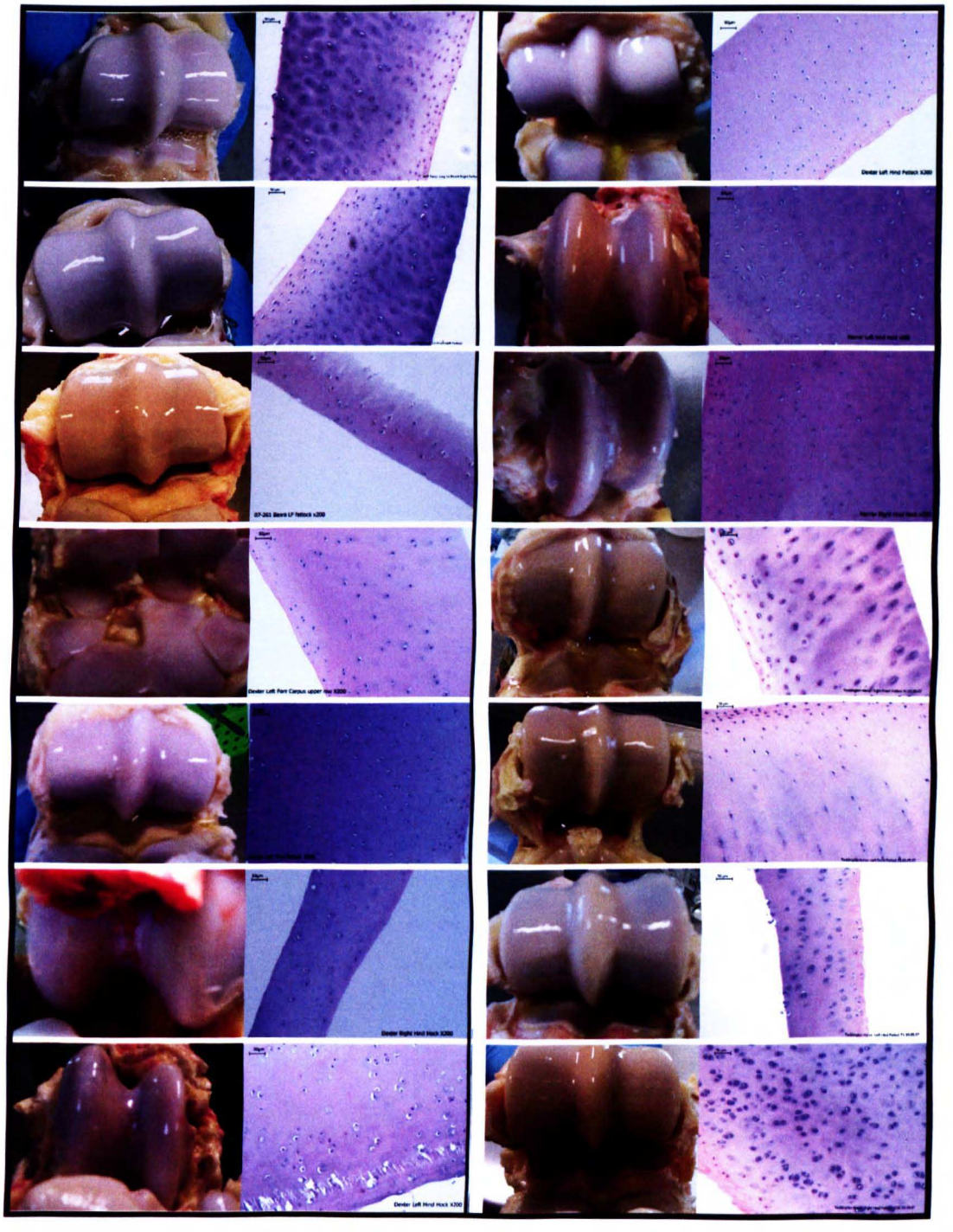


Appendix 4.

Representative histological images of samples used to show macroscopically intact cartilage, plate 1

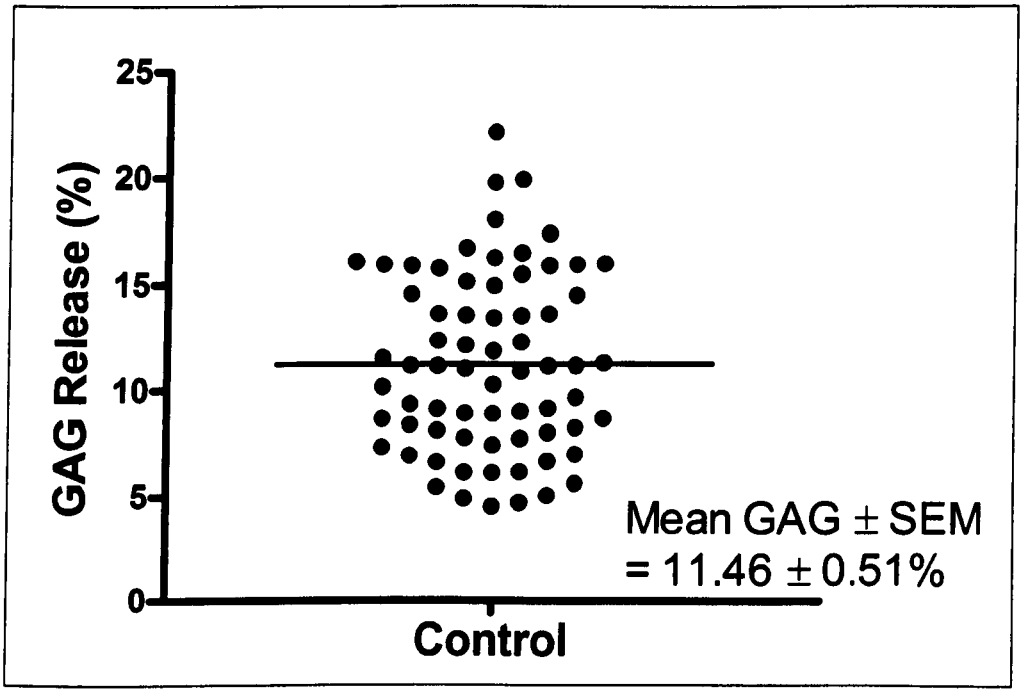


Representative histological images of samples used to show macroscopically intact cartilage, plate 2



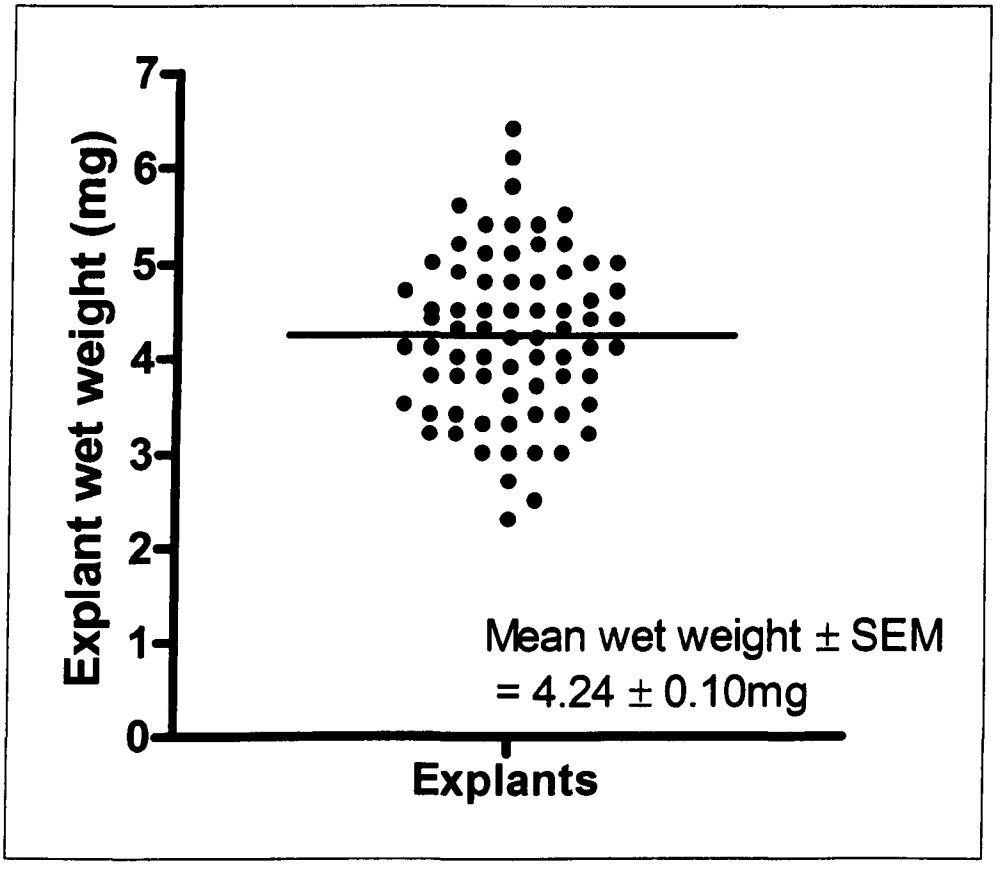
Appendix 5.

Mean percentage of glycosaminoglycan (GAG) release from control explants from 14 horses used in chapter 2 (66 values)



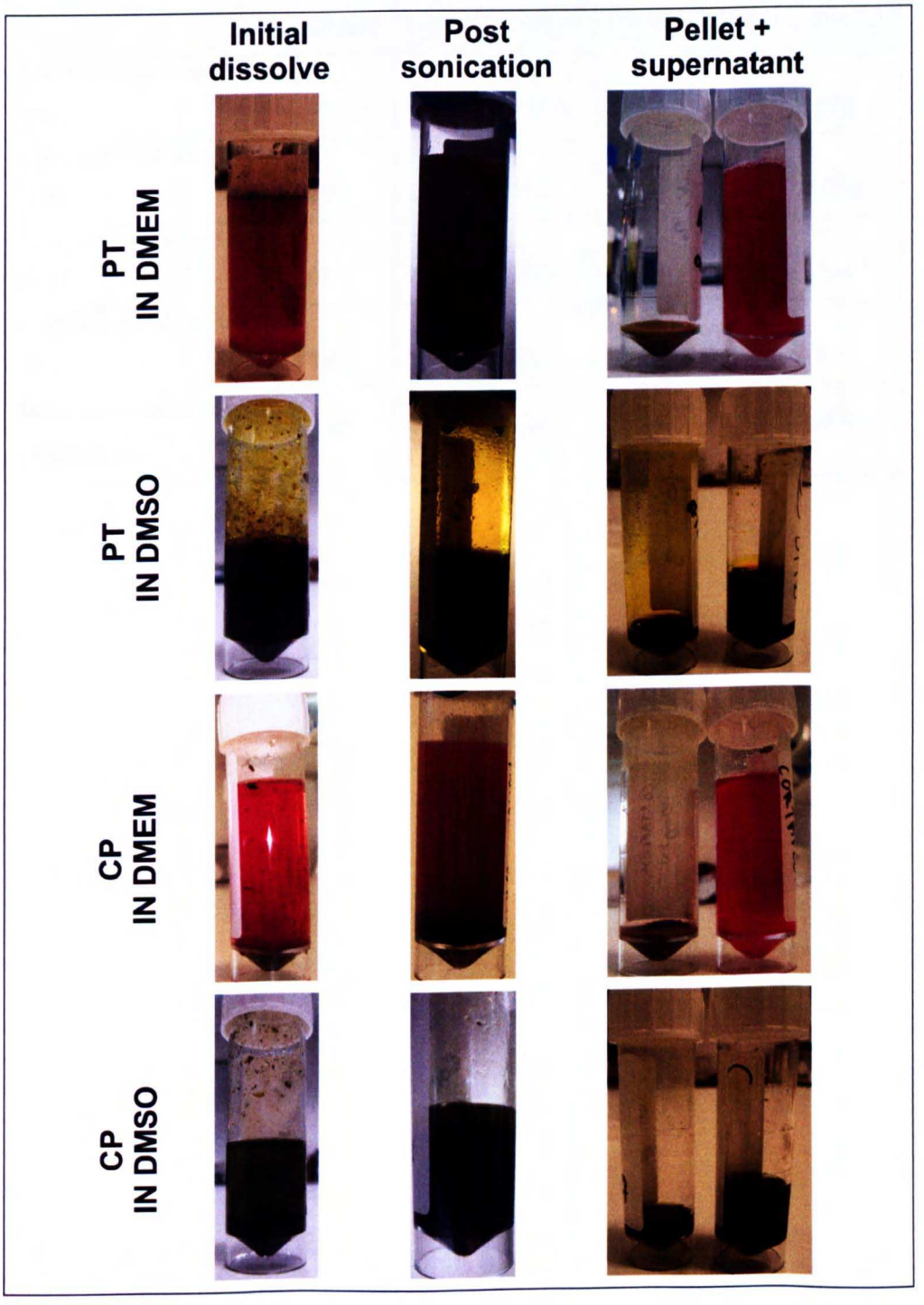
Appendix 6.

Mean explant wet weights (mg) from 72 explants



Appendix 7.

Solubilising the plant extract cocktail (PT) and commercial product (CP) in Dulbecco's modified Eagle's medium (DMEM) and dimethyl sulfoxide (DMSO)



Appendix 8.

Calculations for determining degree of dissolution of materials tested in Chapter 4

	PT in DMEM	CP in DMEM	PL in DMEM	PT in DMSO	CP in DMSO	PL in DMSO
End material weight (mg)	134	120.4	120.8	431	697.3	676
Start material weight (mg)	200	200	200	1000	1000	1000
Quantity in solution (mg)	66	79.6	79.2	569	302.7	324
Final volume of solution (ml)	19.3	18.2	18	8.5	7.6	8.1
Final concentration (mg/ml)	**3.42**	**4.37**	**4.40**	**66.94**	**39.83**	**40.00**

Appendix 9.

Native glycosaminoglycan (GAG) content of the extracts tested in chapter 4, including the plant cocktail (PT), the commercial product (CP) and placebo (PL) dissolved in either Dulbecco's modified Eagle's medium (DMEM) or dimethyl sulfoxide (DMSO). Maximum concentration tested on explants in chapter 4 was 400µg/ml. All samples were tested as run in the GAG assay. Values under 10µg/ml or above 70µg/ml are off the standard curve and are inaccurate.

Samples	Sample GAG (µg)
PT DMEM 400µg	0.0
PT DMSO 400µg	11.4
CP DMEM 400µg	1.0
CP DMSO 400µg	0.0
PL DMEM 400µg	0.0
PL DMSO 400µg	0.0

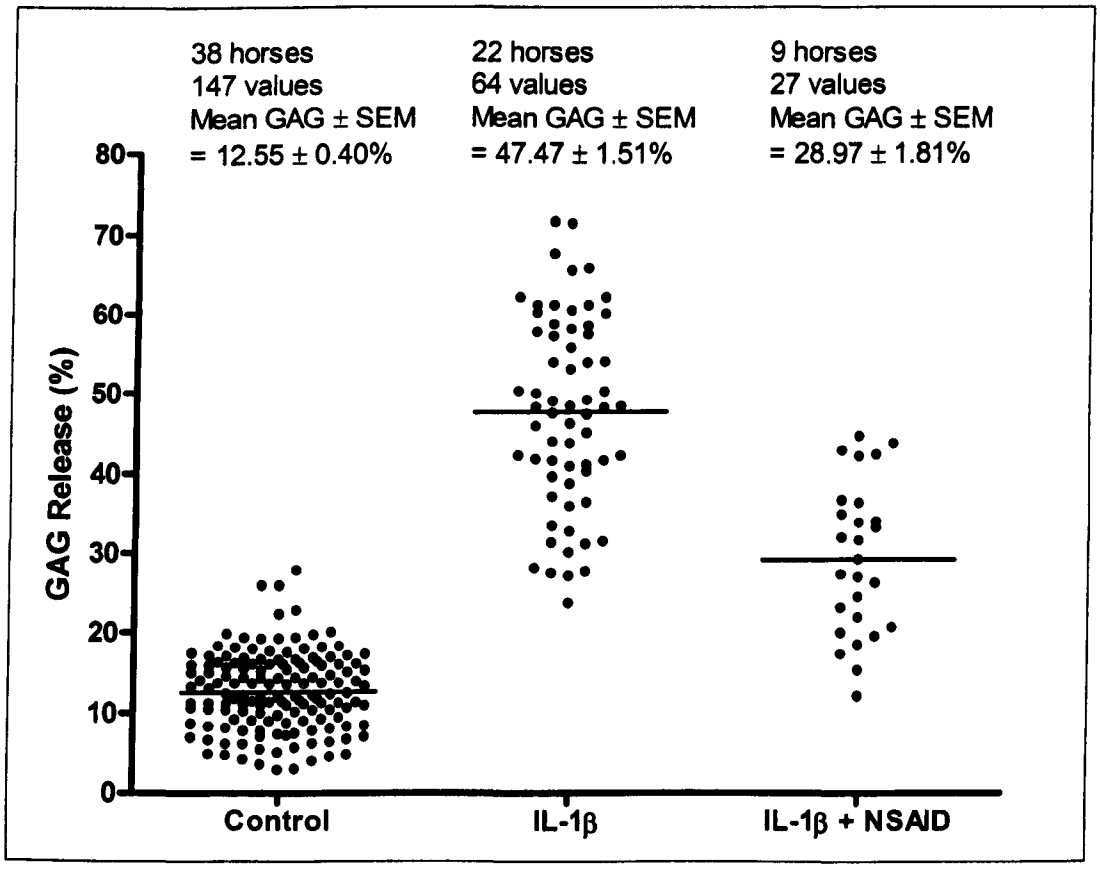
Appendix 10.

BLAST sequence data for the antibodies used in this thesis. Peptide sequences of the antibodies were checked using the NCBI Basic Local Alignment Search Tool (Protein BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

Antibody	Company + Catalogue #	Immunogen	Antibody	Uni Prot accession # for BLAST search	*Equus caballus* Protein	% sequence identity of equine to human
CILP	R&D Systems AF4477	Recombinant human CILP-1	Sheep polyclonal	O75339 αα 724-1184	Predicted: CILP	100%
CLUS-α	Santa Cruz Sc-6419	Peptide mapping at the C-terminus of clusterin-α of human origin	Goat polyclonal	P10909 αα 228-449 (α subunit sequence)	Clusterin precursor CLUS_HORSE	100%
					Predicted: retinal clusterin-like protein CLUL1b	75%
MMP-1	Abcam Ab38929	Synthetic peptide based on the hemopexin domain of the human MMP-1 sequence	Rabbit polyclonal	P03956 αα 284-466 (the 4 hemopexin domain sequences)	Interstitial collagenase precursor MMP1_HORSE	100%
MMP-3	Abcam Ab18898	Recombinant full length protein	Goat polyclonal	P08254 (full sequence)	Stromelysin-1 precursor MMP3_HORSE	100%
					Interstitial collagenase precursor MMP1_HORSE	95%
					Predicted: similar to MMP12	100%
TSP	Abcam Ab1823	Reduced and alkylated form of human TSP-1	Mouse monoclonal	P07996 (full sequence)	Predicted: Thrombospondin 1 TSP1_HORSE	99%
					Thrombospondin 2 TSP2_HORSE	97%
					Cartilage oligomeric matrix protein COMP_HORSE	53%
					Predicted: similar Thrombospondin 3 TSP3_HORSE	54%
TSP-1	Santa Cruz Sc-73158	Reduced and alkylated form of human TSP-1	Mouse monoclonal	P07996 (full sequence)	Predicted: Thrombospondin 1 TSP1_HORSE	99%
					Thrombospondin 2 TSP2_HORSE	97%
					Cartilage oligomeric matrix protein COMP_HORSE	53%
					Predicted: similar Thrombospondin 3 TSP3_HORSE	54%

Appendix 11.

Global overview of the mean percentage of glycosaminoglycan (GAG) release from cartilage explants incubated in culture medium alone (control), recombinant equine IL-1β (10ng/ml), or recombinant equine IL-1β (10ng/ml) + Carprofen, a nonsteroidal anti-inflammatory drug (NSAID; 100μg/ml). Data plots represent results from cartilage explants from multiple horses from multiple experiments.



Appendix 12.

Manufacturer's addresses

Abcam plc
330 Cambridge Science Park,
Cambridge,
Cambridgeshire
CB4 0FL
UK

BD Biosciences
21 Between Towns Road
Cowley
Oxfordshire
OX4 3LY
UK

Bio-Rad Laboratories
Bio-Rad House
Maxted Road
Hemel Hempstead
Hertfordshire
HP2 7DX
UK

Bruker Daltonics Inc
40 Manning Road,
Manning Park,
Billerica,
Massachusetts
01821
USA

Bruker UK Ltd
Banner Lane,
Coventry,
CV4 9GH
UK

Dako UK Ltd
Cambridge House,
St Thomas Place,
Ely,
Cambridgeshire
CB7 4EX
UK

Dionex UK Ltd
4 Albany Court,
Camberley,
Surrey
GU16 7QL
UK

Equine America UK
7 Lawson Hunt Business Park,
Broadbridge Heath,
West Sussex
RH12 3JR
UK

Fluka
Fluka Chemie AG
9471 Buchs
SWITZERLAND

GE Healthcare
Pollards Wood,
Nightingales Lane,
Chalfont St Giles,
Buckinghamshire
HP8 4SP
UK

GraphPad Software Inc
2236 Avenida de la Playa,
La Jolla,
California
92037
USA

Heraeus-Christ
Osterode
GERMANY

ImageJ
U. S. National Institutes of Health,
Bethesda,
Maryland
USA

Invitrogen Ltd
3 Fountain Drive,
Inchinnan Business Park,
Paisley,
Renfrewshire
PA4 9RF
UK

Leica Microsystems UK Ltd
Davy Avenue,
Knowlhill,
Milton Keynes,
Buckinghamshire
MK5 8LB
UK

MARS Horsecare UK Ltd
29 Old Wolverton Road,
Milton Keynes,
Buckinghamshire
MK12 5PZ
UK

National Diagnostics UK Ltd
Unit 4,
Fleet Business Park,
Itlings Lane,
Hessle,
East Riding of Yorkshire
HU13 9LX
UK

Park Tonks Ltd
48 North Road,
Great Abington,
Cambridge
CB21 6AS
UK

Pfizer
Ramsgate Road,
Sandwich,
Kent
CT13 9NJ
UK

Promega UK Ltd
Delta House,
Southampton Science Park,
Southampton,
Hampshire
SO16 7NS
UK

R & D Systems Europe Ltd
19 Barton Lane,
Abingdon Science Park,
Abingdon,
Oxfordshire
OX14 3NB
UK

Roche Diagnostics Ltd
Charles Avenue,
Burgess Hill,
West Sussex
RH15 9RY
UK

Santa Cruz Biotechnology Inc
Bergheimer Str. 89-2,
69115 Heidelberg,
GERMANY

Sigma-Aldrich Company Ltd
The Old Brickyard,
New Road,
Gillingham,
Dorset
SP8 4XT
UK

Thermo Fisher Scientific UK Ltd
Bishop Meadow Road,
Loughborough,
Leicestershire
LE11 5RG
UK

Thermo LabSystems
St. Georges Court,
Hanover Business Park,
Altrincham,
Cheshire
WA14 5TP
UK