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Effects of Resveratrol, Piceatannol and Quercetin in an Equine Cartilage Explant Model for Potential Use in Treating Osteoarthritis

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Declaration

I hereby declare that except where specific reference is made to the work of others, the content of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other University. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. This dissertation contains fewer than 47,000 words including appendices, references, footnotes, tables and figures and has 90 figures and 223 tables.

Purba Islam

Abstract

Currently there are no disease-modifying osteoarthritis drugs (DMOADs) available and the treatment for osteoarthritis (OA) is still based on minimising symptoms. Commonly used drugs for OA have health hazards from long and short term use and has led to patients using alternatives therapies such as nutraceuticals.

In this study, three natural phenols (resveratrol, piceatannol and quercetin) were compared with commonly used drugs used for treating OA; phenylbutazone (Non-steroidal anti-inflammatory drug, NSAID) and prednisolone (corticosteroid) and a nitric oxide inhibitor (S-ethylisothiurea) in an IL-1 β stimulated equine cartilage explant culture. Treatments at 100 μ g/ml were assessed for their ability to inhibit inflammatory markers present in OA (nitric oxide, PGE₂, glycosaminoglycan, cell viability). Resveratrol, piceatannol and quercetin showed close to 100% nitric oxide inhibition similar to S-ethylisothiurea. Piceatannol was the only treatment which showed significant glycosaminoglycan inhibition. Cell viabilities for fresh cartilage, cultured control cartilage, piceatannol (in the presence of IL-1 β) and resveratrol (in the presence of IL-1 β) treated cartilage were 83.9 $\pm$ 0.98 %, 76.8 $\pm$ 2.59 %, 78.8 $\pm$ 3.57 % and 63.1 $\pm$ 4.35 % respectively.

In dose-response IL-1 β stimulated cartilage explant studies, piceatannol and quercetin significantly inhibited nitric oxide at a concentration range of 1-300 μ g/ml, whereas for resveratrol it was 10-300 μ g/ml. Effective inhibition of GAG breakdown for resveratrol and quercetin was in a range of 10-30 μ g/ml in the stimulated cartilage explant. The IC₅₀ for inhibition by piceatannol of GAG breakdown was 2 μ g/ml. Inhibition of PGE₂ production appeared to be obscured by the vehicle (1% DMSO) used to dissolve the phenols.

This study suggests that nitric oxide is not playing a major role in GAG breakdown and cell death in the stimulated equine cartilage explant. Moreover, in this study, it was observed that GAG breakdown does not appear to initiate cell death at least at the early stages of an OA-like condition.

Resveratrol, piceatannol and quercetin may be potential DMOAD candidates to treat OA. Further studies on their beneficial effects, especially *in vivo*, should be compared against novel OA-modulators

(such as GLPG1972) which are currently in clinical trials as they may offer a far cheaper therapeutic alternative.

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All praises by the name of All Mighty.

List of abbreviations

Abbreviation	Meaning
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGRG	Alanine-Arginine-Glycine-Serine
ANOVA	One-way analysis of variance
ATP	Adenosine Triphosphate
CO	Control
COX	Cyclooxygenase
CS	Chondroitin sulfate
DMM	Destabilization of the medial meniscus
DMMB	Dimethylmethylene blue
DMOADs	Disease-modifying osteoarthritis drugs
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EIT	S-ethyl isothiurea
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EU	Endotoxin units
GAG	Glycosaminoglycan
HIF	Hypoxia-inducible factor
Histo	Histology
H&E	Haemotoxylin & Eosin
IC₅₀	Half maximal inhibitory concentration
IL	IL-1 β
iNOS	Inducible nitric oxide synthase
KS	Keratin sulfate
LPS	Lipopolysaccharide

MCP joint	Metacarpophalangeal joint
MMP	Metalloproteinase
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBS	Phosphate buffered saline
PBZ	Phenylbutazone
PCTL	Piceatannol
PGs	Proteoglycans
PGE₂	ProstaglandinE ₂
PRED	Prednisolone
PMB	Polymyxin B
QH	Quercetin high dose
QL	Quercetin low dose
RO	Reverse osmosis
RSV	Resveratrol
SLRPs	Small leucine-rich proteoglycans
SOFG	Safranin O and fast green
STAT	Signal transducer and activator of transcription
VC	Vehicle control
5-LOX	5-lipoxygenase

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Chapter 1

Introduction

1.1 Osteoarthritis

Osteoarthritis (OA) is one of the most common degenerative joint diseases and is characterized by the degradation of articular cartilage and inflammation of the joint (Taruc-Uy and Lynch, 2013). OA is described as a heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone and at the joint margins (Altman *et al.*, 1986). There is an imbalance that occurs between repair and degradation of articular cartilage in OA (Lotz, 2010). In 1994 The National Institute of Arthritis, Musculoskeletal, and Skin Diseases, The National Institute on Aging, The Arthritis Foundation and The Orthopaedic Research and Education Foundation, redefined OA as: 'a group of overlapping distinct diseases which may have different aetiologies, but with similar biologic, morphologic, and clinical outcomes. Disease processes do not only affect the articular cartilage but also involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane, and peri-articular tissues. Ultimately the articular cartilage degenerates with fibrillation, fissures, ulceration, and full thickness loss of the joint surface' (Brandt *et al.*, 2008).

1.2 Equine OA

Equine degenerative arthritis was first reported in 1938 and at that time pathological changes were compared with human OA. Examination of osteoarthritic joints was initially limited to morphological observations (Callender and Kelser, 1938). In 1966 equine OA received its first clinical attention at the 'American Association of Equine Practitioners'. At the same time its relationship with lameness and 'use trauma' became a central etiologic concept (McIlwraith *et al.*, 2012). By 1975 articular cartilage lesions were considered the essential criteria of OA but it was also well understood that they may not be the necessarily important cause of clinical disease. Today equine OA may be expressed as a group of disorders described by a common end stage: progressive deterioration of the articular cartilage accompanied by changes in the bone and soft tissues of the joint (McIlwraith *et al.*, 2012). Spontaneous joint disease is a common clinical problem in the horse. It has been estimated that up to 60% of lameness is related to OA (McIlwraith *et al.*, 2012). The metacarpophalangeal (MCP) joint is the most common joint for spontaneous OA in the racehorse due to its close-fitting articular surfaces which can quickly develop linear erosions and wear lines in association with osteochondral fragmentation (McIlwraith *et al.*, 2012; Riggs, 2010). Other than racing horses, an age-related osteoarthritic process is naturally

present in both wild and domesticated horses (Cantley *et al.*, 1999; Pool, 1996). Among all lesion sites, the fetlock is thought to be one of the main causes of lameness in athlete horses (Vanderperren and Saunders, 2009). The most possible reason of maximum injury is the repetitive high-speed exercise resulting in cyclical impact loading of a joint. After each cycle of load there is a lag phase as the cartilage repossesses its relaxed dimensions which is correlated with redistribution of fluid within the matrix (Mow and Hung 2003), and a high rate of cyclical loading, as achieved by the galloping horse, may interfere with full function of this mechanism. Equally, there are reparative mechanisms that take days or weeks to complete (Burr, 2003; Hunziker and Tyler, 2003).

1.3 Metacarpophalangeal joint

The metacarpophalangeal joint, also known as the fetlock, consists of the distal extremity of the third metacarpal bone (III MC), first phalanx (P1), proximal sesamoid bones (PSB) and the intersesamoidean ligament, which connects the PSB along their axial aspect (Dyson, 2007; Riegel and Hakolla, 2011) (Figure 1.1).

The key components of the extracellular matrix (ECM) of articular cartilage are collagen, proteoglycans (PGs), and water (Figure 1.2). Water content may vary from 70% to 80%, according to age. The other components are approximately 50% (collagen) and 35% (PGs)

on a dry weight basis. The remaining 15% consists of about two thirds (10% of total dry weight) glycoproteins (substances such as proteinases and inhibitors of these, growth factors, specific molecules such as fibronectin, lubricin, cartilage oligomeric protein [COMP]), a small amount of minerals (3%), lipids (1%), and miscellaneous components (1%) are also present. In articular cartilage, the proteoglycan is present in a characteristic network incorporated between the collagen fibrils in the interterritorial matrix (Poole *et al.*, 1982). The

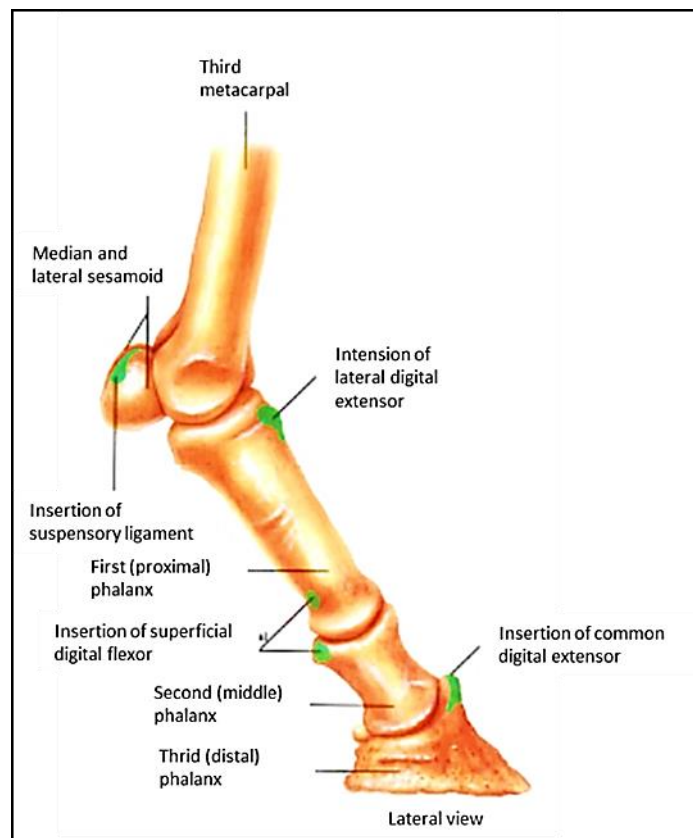


Figure 1.1: Metacarpophalangeal joint (lateral view) (Riegel and Hakolla, 2003)

nature of proteoglycans depends upon the structure of both the protein and glycosaminoglycan elements. Earlier the glycosaminoglycans were termed mucopolysaccharides. The glycosaminoglycans may be classified as being sulfated or non-sulfated and only the sulfated glycosaminoglycans being part of a proteoglycan (Jackson *et al.*, 1991). Five sulfated glycosaminoglycans are commonly determined: chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin. However, dermatan sulfate bears a biosynthetic relationship to chondroitin sulfate, and heparin bears a similar relationship to heparan sulfate. In contrast, there is only one non-sulfated glycosaminoglycan which is hyaluronic acid (Laurent and Fraser, 1992). All the glycosaminoglycans are present in articular cartilage other than heparin (Roughley and Lee, 2018). The cellular component of articular cartilage is relatively small which is approximately 1% to 12% volume percentage, depending on the location within the joint and the depth in relation to the surface (Weeren, 2016).

1.1 Etiology of OA

The MCP joint is especially susceptible to osteoarthritis and injuries as it is a high-motion condylar joint and also receives high loads

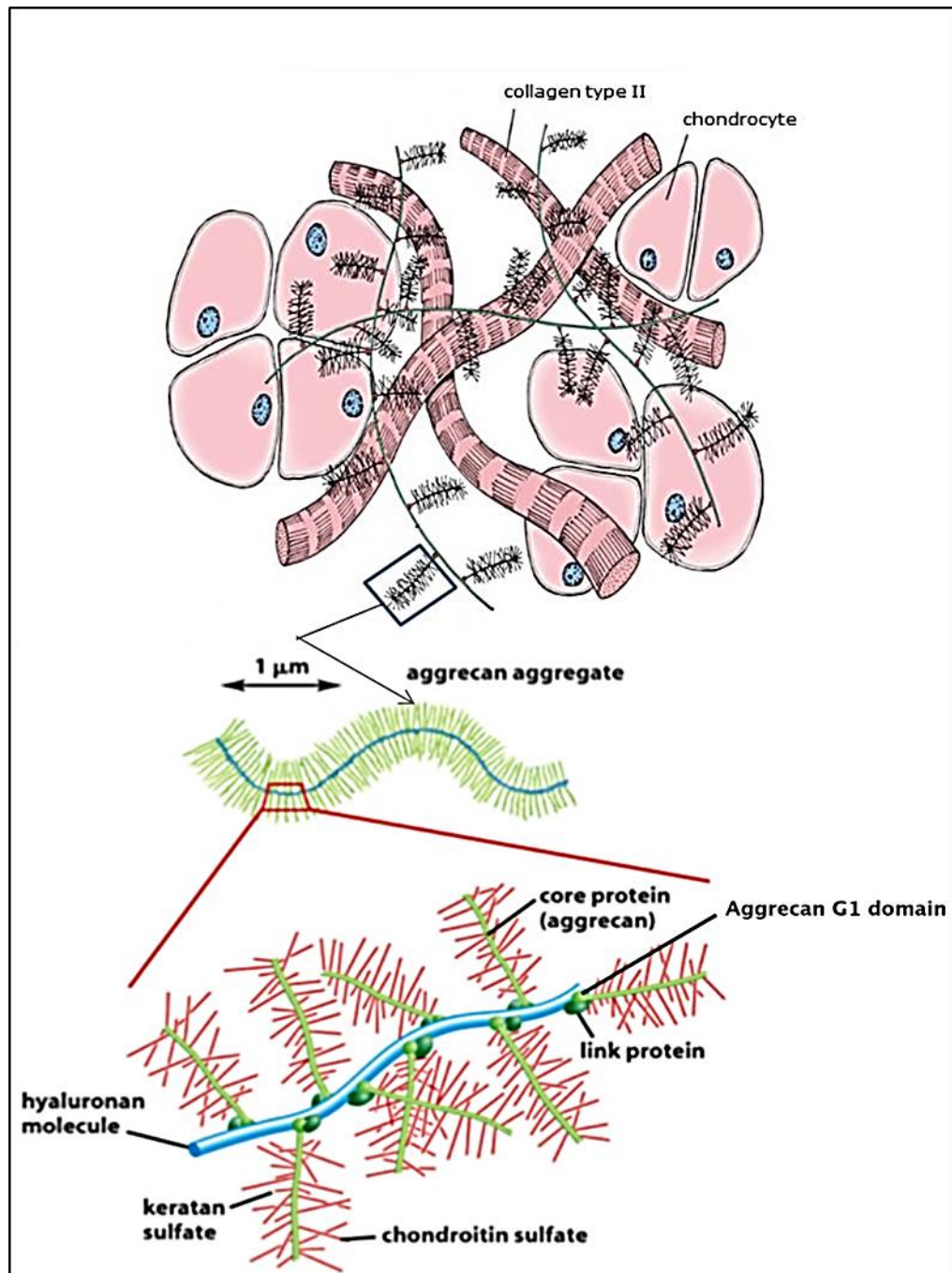


Figure 1.2: Representation of the microscopic structure of cartilage. Arrow indicates large aggrecan aggregate with an enlarged area depicting aggrecan molecules consisting of a core protein, keratan and chondroitin sulfate GAGs linked via the G1 globular domain to the hyaluronic acid monomer. Adapted from Erwin & Hood (2014); Ross and Pawlina (2011).

during racing. All types of articular injuries may lead to OA (Neundorff *et al.*, 2010). OA mechanically causes disorder in which the

consequences of abnormal joint mechanics aggravate biological effects which are mediated biochemically— for example, through cytokines, matrix-degrading enzymes and toxic oxygen radicals. OA develops in one of the following ways or in a combination of the two (Figure 1.3): (1) the biomaterials that consist of various connective tissues of the joint are normal, but the mechanical stresses on the joint are excessive; (2) the loads lodged on the joint tissues are normal but the biomaterials abnormal (Brandt *et al.*, 2006).

1.1.1 Abnormal loading on normal cartilage

Abnormal or excessive loading of joints is considered as one of the causes of OA development. Borrelli *et al.* (1997) established the significant effects of a single high impact load on articular cartilage *in vitro*. Within the study, destruction of cartilage in the area of direct impact was examined radiographically, histologically and by scanning electron microscopy. Moreover, prostaglandin (PG) synthesis was significantly decreased in the area of direct impact. These traumatic observations are especially applicable to horses, as recurrent concussive forces combine with their athletic work and their anatomical design. Furthermore, when galloping, the total weight of the animal is transmitted through a single MCPJ joint

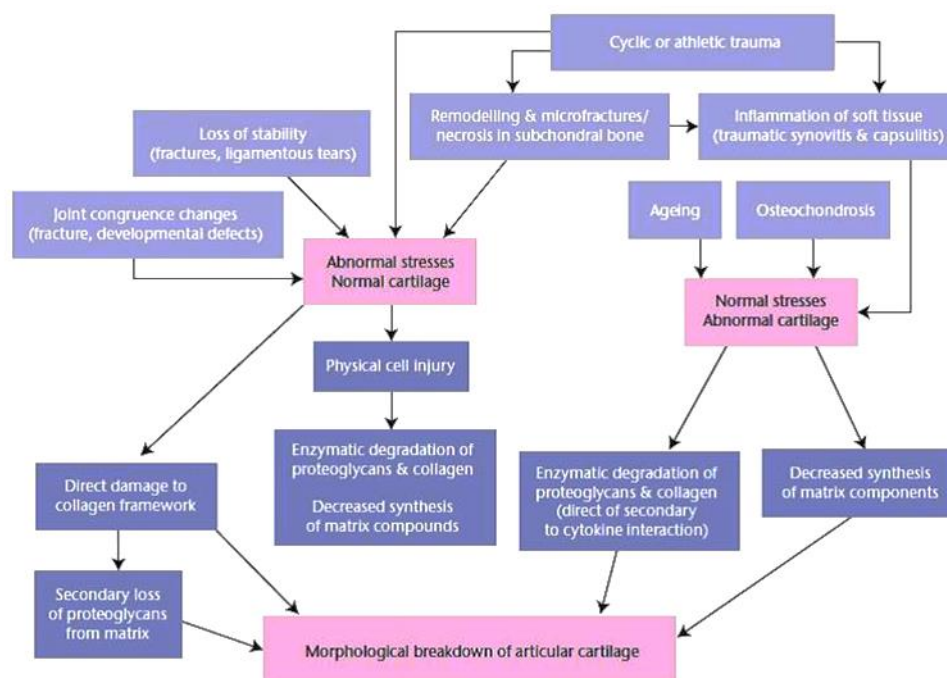


Figure 1.3: Possible pathways for degradation of articular cartilage secondary to joint trauma in the horse (McIlwraith *et al.*, 2012)

during each stride. Therefore, equine joints (especially the MCPJ) are repeatedly exposed to excessive loading forces at high speeds. This predisposes equine cartilage towards traumatic OA (Murray *et al.*, 1999). Performance horses are vulnerable to OA as extreme exercise increases the loading of joints. This effect is exaggerated during states of physiological fatigue. Under such condition, musculotendinous units exhibit a reduced ability to absorb the loads associated with galloping (Radin, 1999). Thus, the biomechanical stress exerted on joints and articular cartilage becomes exaggerated. For these reasons, equine OA is common in young, athletic animals (McIlwraith and Trotter, 1996).

1.1.2 Normal loading on abnormal cartilage

Another factor which can predispose equines toward OA formation is the presence of abnormal cartilage, even when subjected to normal loading forces. Structurally compromised cartilages are considered as abnormal cartilage. This potentiates physical damage to the collagen framework and also exposes chondrocytes to deleterious physical forces. Consequently, changes in chondrocyte metabolism and mechanical injury to cartilage develop, thus stimulating the development of traumatic OA. Age is thought to be one of the factors to predispose individuals towards the development of abnormal cartilage. In aged chondrocytes there is abnormal secretion, reduced cellular density, and impaired defence mechanisms at the cellular level (Lotz and Loeser, 2012). Moreover, a decrease in anabolic activity within aged cartilage has also been observed (Aigner *et al.*, 2007). This may be due to a decrease in responsiveness to insulin-like growth factor-1 (IGF-1), a key anabolic cytokine, within aged chondrocytes (Fortier and Miller, 2006). IGF-1 has been observed to both decrease cartilage degradation and increase the synthesis of matrix macromolecules *in vitro* (Tyler, 1989). Moreover, IGF-1 has shown its ability to protect chondrocytes from apoptosis (Higgins and Johnson, 2010). Some other factors such as genetic tendency may contribute towards the formation of abnormal cartilage (Valdes

and Spector, 2010). Furthermore, obesity may also play a role via increasing the amount of circulating adipokines such as leptin (Poonpet and Honsawek, 2014). Pre-existing conditions such as septic arthritis and osteochondrosis may also facilitate equines towards OA development (McIlwraith and Trotter, 1996). This predisposition may be explained by an increase in inflammatory cytokine activity observed in such conditions, thus indicating an important inflammatory role in OA (Alwan *et al.*, 1991).

1.2 Inflammation and OA

Is OA an inflammatory disease? Historically, the answer is no: however, there is a lot of evidence that inflammatory components may be found in some patients at some phases of the disease. For example, synovial histology may demonstrate noticeable synovial hyperplasia and a dense mononuclear cell infiltrate (Haraoui *et al.*, 1991). The inflammation is focal and most notable where the synovium is close by cartilage. Recently, expression of oncoproteins and NF- κ B, an essential transcription factor for the expression of a variety of proinflammatory genes, has also been found present in OA synovium (Marok *et al.*, 1996; Roivainen *et al.*, 1996). *In vivo*, leucocytes are observed to migrate into OA knee joints (Jones *et al.*, 1991). In the case of OA progression systemic markers of

inflammation like C reactive protein are increased in many cases (Spector *et al.*, 1997).

1.3 Mechanism of OA

Normal adult articular cartilage consists of extracellular matrix (water, collagen, proteoglycans and a very small component of calcium salt) and chondrocytes (Goldring and Marcu, 2009). The turnover rate of collagen is relatively slow, whereas proteoglycan turnover is rapid (Mow *et al.*, 2005). The normal turnover of this matrix components is mediated by the chondrocytes, which synthesize these components and the proteolytic enzymes responsible for their breakdown. Chondrocytes are, in turn, influenced by a number of factors, including polypeptide growth factors and cytokines, structural and physical stimuli and even the components of the matrix itself (Wise, 2010). Humoral factors such as cytokines can modulate connective tissue metabolism and their involvement in the pathophysiology of osteoarthritis is of significant interest. At least three cytokines, interleukin (IL-1 β), tumor necrosis factor (TNF α), and interleukin-6, were identified in articular tissue and thought to play a role during inflammation (Pelletier *et al.*, 1993) (Figure 1.4). Cytokines also prohibit chondrocyte compensatory synthesis pathways which are required to restore the integrity of the degraded extracellular matrix (ECM) (Fernandes *et al.*, 2002)

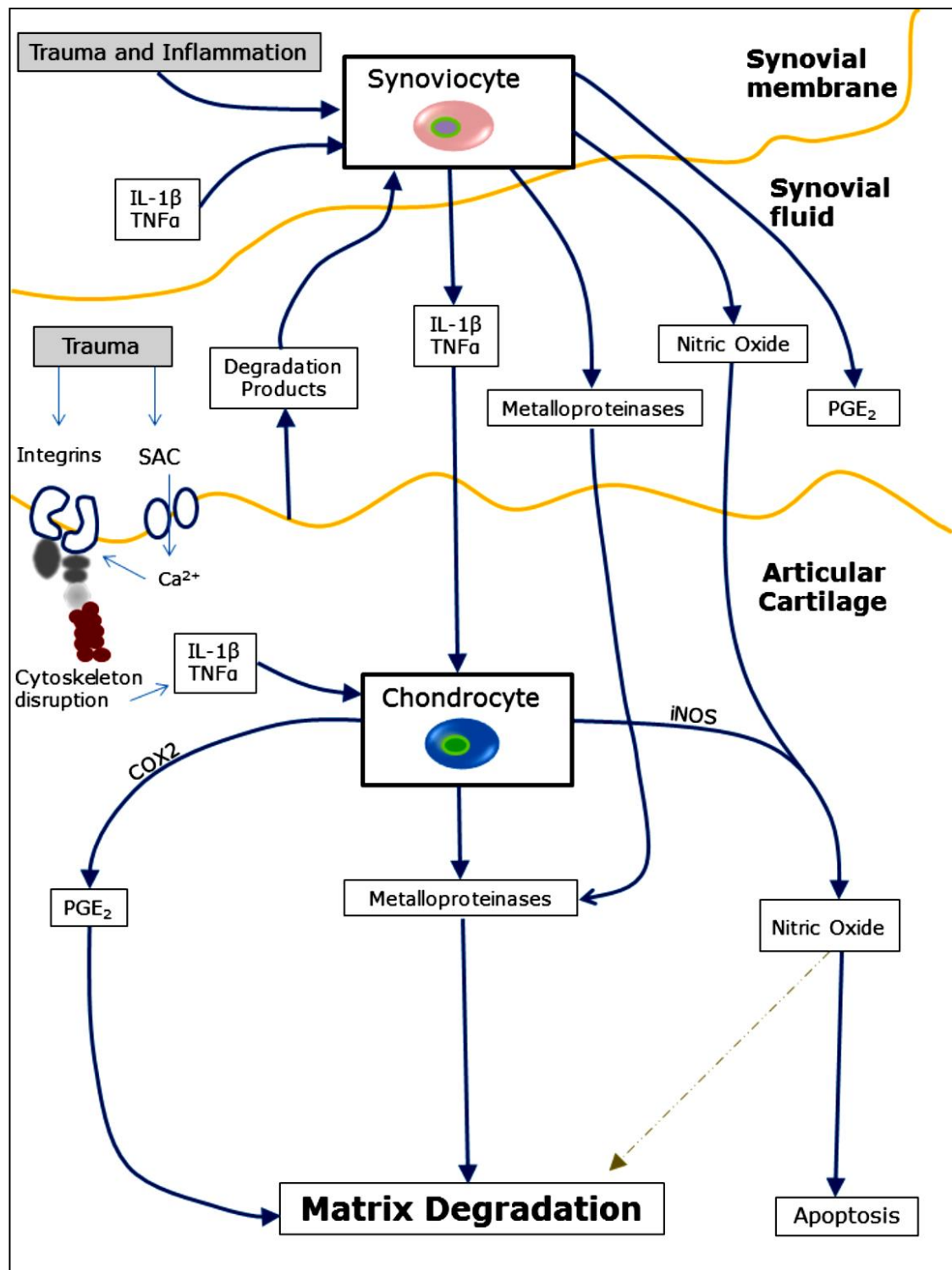


Figure 1.4: Overview of the factors involved in the enzymatic degradation of articular cartilage. SAC = stretch activated ion channel. Adapted from (Wan *et al.*, 2012, McIlwraith and Trotter, 1996).

(Figure 1.4). Current research shows that osteoarthritis progression involves a complex network of biochemical factors, including proteolytic enzymes, that lead to a breakdown of the cartilage macromolecules (Fernandes *et al.*, 2002).

1.3.1 Interleukin-1 β

Interleukin 1 β (IL-1 β) is a pleiotropic pro-inflammatory cytokine which plays a critical role in the development of OA. It was found that chondrocytes release proinflammatory cytokines, chemokines, neutral metalloproteinases (MMPs), and nitric oxide (NO) in response to IL-1 β (Fernandes *et al.*, 2002). These inflammatory mediators develop pathogenesis of OA. Synthesis of IL-1 β is mediated by NF- κ B, as is the expression of cyclooxygenase 2 (COX-2) (Tak *et al.*, 2001). IL-1 β treatment markedly increased the production of NO and PGE₂, as well as increases the expression of iNOS and COX-2 in chondrocytes (Zhou *et al.*, 2016). COX-2 is an important mediator of pain and inflammation in osteoarthritic joints, mainly stimulated by IL-1 β . After induction, COX-2 participates in the sequential enzymatic reactions which help in the synthesis of PGE₂. PGE₂ plays a critical role in the pathophysiology of OA (Hardy *et al.*, 2002; Kojima *et al.*, 2004). IL-1 β triggers chondrocytes to

release proteolytic enzymes and MMPs, which are key regulators of cartilage destruction (Klatt *et al.*, 2009).

1.3.2 Metalloproteinases

Cytokines such as IL-1 β and TNF- α originated from activate synoviocytes, mononuclear cells or articular cartilage itself and significantly up-regulate metalloproteinase (MMP) gene expression. IL-1 β and TNF- α markedly up-regulate MMP-3 steady-state mRNA derived from human synovium and chondrocytes. The inhibition of IL-1 β and/or TNF- α up-regulation of MMP gene expression shows a logical development in the potential medical therapy of OA (Fernandes, *et al.*, 2002). Osteoarthritic chondrocytes also show a higher sensitivity to stimulation by IL-1 β with respect to metalloprotease production compared to normal chondrocytes and seems to be related to an increase in the level of IL-1 β receptors (Pelletier *et al.*, 1993).

1.3.3 PGE₂

It has recently been shown that high levels of PGE₂ are produced by OA tissues when stimulated by IL-1 β , which might have a role in the degeneration of bone and cartilage associated with OA (Hardy *et al.*, 2002). It was also found that the nitric oxide-induced death of OA chondrocytes was intensified through the production of PGE₂ by the

chondrocytes themselves (Notoya *et al.*, 2000). It is known that in the PGE₂ synthesis pathway, cyclooxygenase (COX) is a key enzyme that metabolizes arachidonic acid to PGG₂ and PGH₂. There are two isoforms of COX and they are designated as COX-1 and COX-2 (Kujubu *et al.*, 1991; Xie *et al.*, 1991). COX-1 is present in various cells and tissues and is important in maintaining homeostasis. Whereas COX-2 is induced in inflammatory cells and tissues by various stimuli including cytokines. It is suggested that COX-2 has a key role in the process of inflammation. Increased PGE₂ production in response to stimulation by proinflammatory cytokines is accompanied by the upregulation of COX-2 expression. PGE synthase (PGES) acts downstream of COX which helps to catalyse the conversion of PGH₂ into PGE₂ (Kudo I 1999). At least three forms of human PGES have been characterized, such as membrane-associated PGES (mPGES)-1 (Jakobsson *et al.*, 1999), mPGES-2 (Tanikawa *et al.*, 2002), and cytosolic PGES (cPGES) (Tanioka *et al.*, 2000). Among these, mPGES-1 was originally known as microsomal glutathione S-transferase 1-like 1 (MGST1-L1), and it is a glutathione-dependent enzyme which shows coordinated induction with COX-2 by inflammatory stimuli in various cells and tissues (Jakobsson *et al.*, 1999; Tanioka *et al.*, 2000). Kojima *et al.* (2004) reported that mPGES-1 is a key enzyme in the regulation of PGE₂ production in cytokine stimulated chondrocytes from patients with OA. They also observed that mPGES-1, located downstream of COX-

2, is induced in chondrocytes by cytokine stimulation and the expression of mPGES-1 and COX-2 was markedly induced by IL-1 β in parallel with an increase in PGE₂ synthesis. mPGES-1 is a member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) family, which also contains 5-lipoxygenase-activating protein and leukotriene C4 synthase. Co-localization of mPGES-1 and COX-2 might be related to the preferential increase of PGE₂ production after IL-1 β stimulation due to functional linkage between these two enzymes in chondrocytes. This coordinated expression of mPGES-1 and COX-2 over time might sustain higher PGE₂ production in OA chondrocytes. The induction of mPGES-1 in cytokine-activated chondrocytes and the subsequent increase of PGE₂ production might be important in the pathogenesis of OA. mPGES-1 is a specific enzyme that only catalyses PGE₂ production from PGH₂. mPGES-1 might be an important parameter for the treatment of articular inflammation with cytokine-stimulated conditions in patients with OA (Kojima *et al.*, 2004).

1.3.4 Apoptosis of chondrocytes

Chondrocyte apoptosis and degeneration of articular cartilage are closely correlated. Usually, a high incidence of chondrocyte apoptosis is observed in the severely degenerated cartilage matrix. But it is still unclear whether chondrocyte apoptosis is a cause of or the result of, cartilage breakdown in OA pathogenesis. Degeneration

of the extracellular matrix could contribute to the loss of a survival signal for chondrocytes. Moreover, biological mediators, such as NO, cytokines, or proteinases (e.g., matrix metalloproteinases), from chondrocytes themselves or from other damaged chondrocytes, could directly or indirectly trigger apoptosis. Alternatively, chondrocyte apoptosis could cause the matrix degeneration because chondrocytes are the only cells that exist in the cartilage and maintain the extracellular matrix. Equine OA articular cartilage shows more intense and diffuse positive immunoreactivity to nitrotyrosine than the normal cartilage. In addition, strong positive reactivity was observed in the areas of degenerated extracellular matrix, where frequent chondrocyte apoptosis was noted. It was also observed that the topographical distributions of chondrocyte apoptosis, cartilage matrix degeneration, and NO production almost completely overlapped in the lesional areas of the equine OA articular cartilages which suggest that these pathological phenomena are closely interrelated (Kim *et al.*, 2003). Excessive production of NO has been linked to cartilage catabolism (Häuselmann *et al.*, 1998; Pelletier *et al.*, 1998; Spreng *et al.*, 2000) and chondrocyte apoptosis (Blanco *et al.*, 1998; Hashimoto *et al.*, 1999, 1998).

1.3.5 Depletion of GAG

Cartilage degradation could occur in the absence of instability or traumatic disruption of the tissues and loss of glycosaminoglycan (GAG) is associated with the early morphologic breakdown of the surface of the cartilage and loss of GAG throughout the ECM (Adames *et al.*, 2017). The glycosaminoglycan side chains (GAGs) are an essential component of the proteoglycan molecules (PG) and strongly bind water molecules. The GAGs are capable of functioning as water-binding molecules only when they are intact and bound to the PG core protein. The most abundant GAGs found (in particular with respect to aggrecan) are chondroitin sulfate (CS) and to a lesser degree, keratan sulfate (KS) (Jackson *et al.*, 1991; Laurent and Fraser, 1992). In addition to their mechanical function PGs (notably the small leucine-rich proteoglycan family [SLRPs]) also play pivotal signal transduction roles since they are capable of binding and sequestering growth factors within the ECM (Brown *et al.*, 2012). Hyaluronan (HA) is a glycosaminoglycan that has an important role in the formation of proteoglycan aggregates in the cartilage and is also a component of the synovial fluid. Synovial membrane type B cells and chondrocytes synthesize HA by disaccharide oligomerization (Gadher and Woolley, 1987). Hyaluronan molecules restrict large plasma proteins from entering the synovial fluid but facilitate the passage of small molecules for the maintenance of

nutrition (Goldberg and Buckwalter, 2005). In osteoarthritis, the synthesis of HA is disrupted by increased levels of proinflammatory cytokines, free radicals and proteinases, resulting in HA with a significantly reduced molecular weight, more molecular polydisaccharides and a reduction in synovial fluid viscoelasticity (Balazs & Denlinger 1993). Stimulation of chondrocytes by proinflammatory cytokines increases the production of major cartilage-degrading enzymes, called matrix metalloproteinases and inhibits the synthesis of cartilage proteoglycans and/or type II collagen (Kojima *et al.*, 2004). The MMPs are thought to be important in joint disease as they are implicated in the degradation of the matrix molecules of articular cartilage (Jackson *et al.*, 1991).

1.3.6 Nitric Oxide

Nitric oxide (NO) has been implicated as an important biological mediator of OA. NO production from chondrocytes is significantly higher in OA cartilage compared with that in controls and directly correlates with the severity of OA (Hashimoto *et al.*, 1998, Spreng *et al.*, 2000). The major source of endogenous NO in chondrocytes is inducible nitric oxide synthase (iNOS). Experimental treatment with selective inhibitors against iNOS has significantly reduced the progression of OA *in vivo* (Pelletier *et al.*, 1998, 2000). Excessive endogenous production of NO has been linked to chondrocyte apoptosis (Blanco *et al.*, 1998). NO reacts with the superoxide

radical to form peroxynitrite. Excessive production of NO has been linked to cartilage catabolism (Häuselmann *et al.*, 1998; Spreng *et al.*, 2000) and chondrocyte apoptosis (Blanco *et al.*, 1998; Hashimoto *et al.*, 1998c, 1999). The topographical distributions of chondrocyte apoptosis, cartilage matrix degeneration, and NO production are almost completely overlapped in the lesional areas of the equine OA articular cartilage, suggesting that these pathological phenomena are closely interrelated (Kim *et al.*, 2003).

1.4 Management of OA

So far there is no specific disease modifying osteoarthritis drug (DMOAD). The goals of OA therapy are to decrease pain and maintain or improve joint function. It can be achieved in two ways: non-pharmacological treatment and pharmacological intervention (Lane and Thompson, 1997).

1.4.1 Nonsteroidal anti-inflammatory drugs (NSAIDs)

Cyclo-oxygenase, an enzyme involved in the conversion of arachidonic acid to prostaglandins, exists in two isoforms: COX-1, a constitutive isoform, predominates in the stomach and produces cyto-protective prostaglandins, while COX-2, an inducible isoform predominantly involved in the inflammatory cascade, gives rise to

Table 1.1: Management of OA (Lane and Thompson, 1997).

Therapeutic options in osteoarthritis
Non-pharmacological treatment Physiotherapy (muscle strengthening, and patellar strapping) Occupational therapy (aids and appliances, joint protection) Weight loss Acupuncture Transcutaneous electrical nerve stimulation (TENS)
Pharmacological treatment Simple analgesia Non-steroidal anti-inflammatory drugs COX-2 inhibitors (cyclo-oxygenase-2 selective non-steroidal anti-inflammatory drugs) Topical (non-steroidal anti-inflammatory drugs, capsaicin) Chondroprotective agents
Intra-articular treatment Corticosteroids Hyaluronans

articular pain, swelling and stiffness (Walker-Bone, 2000). Traditionally, COX-1 was thought to be a housekeeping enzyme, required for maintenance of a basal level of PGs to protect gastric mucosa (Cha and DuBois, 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of compounds that provide analgesic (pain-killing) and antipyretic (fever-reducing) effects, and in higher

doses can show anti-inflammatory effects (Ghanghas *et al.*, 2016). Management of osteoarthritis pain is based on a sequential hierarchical approach, with non-steroidal anti-inflammatory drugs (NSAIDs) being the main form of treatment (da Costa *et al.*, 2016). It was reported by da Costa *et al.* (2016) that effective pain relief in OA, but that long-term use is likely to result in gastrointestinal or other complications (Jüni *et al.*, 2006). Some of their side effects—dyspepsia, gastritis, peptic ulcer, gastrointestinal bleeding, haemorrhagic stroke and perforation of gastroduodenal ulcers (Wolfe *et al.*, 1999) are thought to involve defects in the maintenance of intestinal mucosal integrity. NSAIDs also inhibit wound healing, including ulcer healing. The most common clinical manifestations of NSAID related GI damage to tissue are a combination of gastroduodenal erosions and ulcerations, often called NSAID induced gastropathy, affecting 25–50% of chronic NSAID users. NSAID induced gastropathy may limit long term NSAID treatment and causes a significant financial burden on the healthcare system (Richy *et al.*, 2004).

1.4.2 Intra-articular corticosteroids

Intra-articular (IA) corticosteroids are widely used in the management of patients with osteoarthritis of the knee, most commonly in those who have appreciable effusion or other signs of active inflammation (Lane and Thompson, 1997). Glucocorticoids act

directly on nuclear steroid receptors to control the rate of synthesis of mRNA and proteins. This has a number of consequences, including changes in T and B cell functions, changes in white cell traffic, alterations in levels of cytokines and enzymes, and inhibition of phospholipase A₂ resulting in a reduction in pro-inflammatory derivatives of arachidonic acid (Haraoui *et al.*, 1991). Corticosteroids, by inhibiting phospholipase A₂, reduce the production of inflammatory mediators (bradykinin, histamine prostaglandins, leukotrienes, and interleukins 1 and 6) and hence reduce inflammatory pain. There is good evidence to support the judicious use of intra-articular corticosteroids in patients with knee osteoarthritis, but because of multiple intra-articular injections which accelerate cartilage damage, they should not be used as the only treatment of patients with chronic, stable osteoarthritis (Walker-Bone, 2000). Long term use of corticosteroids may exert effects such as anaphylaxis/allergic reaction, joint sepsis, osteonecrosis, post-injection hypo/hyperpigmentation, facial flushing/erythema, Charcot-like arthropathy, hypothalamic–pituitary–adrenal (HPA) axis suppression, fainting and dizziness (McCrum, 2016). Methylprednisolone acetate (MPA), a long-acting corticosteroid (recommended dose of 40 to 100 mg per joint), is advised to aim for the lower end of this dose range (Table 1.2). Doses between 10 and 40 mg have been shown effective inhibition of anti-inflammatory effect maintaining the normal joint

environment (Farquhar *et al.*, 1996; Todhunter *et al.*, 1996). Betamethasone acetate, a medium- to long-acting corticosteroid, has been used in equine medicine for more than 40 years¹⁰⁷ (advised dose is 3 to 18 mg per joint) (Table 1.2) (Houdeshell, 1969). The drug did not have deleterious effects 7 weeks after application in both exercised and unexercised horses in an osteochondral chip model (Foland *et al.*, 1994). However, in vitro work showed suppression of proteoglycan synthesis at low to medium doses (Frean and Lees, 2000). Triamcinolone acetonide, probably the most widely used corticosteroid in equine orthopaedics, has a medium duration of action. It has been also observed that it has potent analgesic action in an LPS-induced lameness model in the horse (Kay *et al.*, 2008).

Table 1.2: Overview of most commonly used corticosteroids for intra-articular use in horses (van Weeren and de Grauw, 2010).

Overview of the most commonly used corticosteroids for intra-articular use			
Name of Drug	Duration of Action	Dosage	Remarks
Methylprednisolone acetate	Long	40–100 mg	The lower end of the dosage range is recommended for an optimal effect while avoiding damage at a longer term
Bethamethasone acetate	Medium to long	3–18 mg	
Triamcinolone acetonide	Medium	6–18 mg	Most commonly used

1.4.3 Nutraceuticals

60 to 90% of arthritis patients adopt complementary and alternative medicine (CAM) in the USA. Among them, chiropractic and herbal therapies are the most common means (Soeken *et al.*, 2003). Moreover, there are different traditional systems (European based plant collections, the Chinese Traditional Medicine, the Kampo system, the Indian Ayurveda, and many more) which are also popular worldwide and have evolved by trial and error. There could be one (or more) active substance(s) within the plant material responsible for pharmacological action which needs to be isolated for the use as a single agent in the context of emerging natural science-based medicine and pharmacy (Fürst and Zündorf, 2014). There are some phytochemicals which have anti-inflammatory properties and may serve to inhibit OA. The Indian spice turmeric (*Curcuma longa*) has been used for centuries in Ayurvedic medicine as an anti-inflammatory agent and its active ingredient is Curcumin. Epigallocatechin-3-gallate, commonly abbreviated as EGCG, is an ingredient of green tea, *Camellia sinensis* has strong anti-inflammatory, antioxidant, anti-infective, anticancer, antiangiogenetic, and chemopreventive effects. Quercetin (2-(3, 4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one) a flavonoid, is a widely spread secondary plant metabolite. Quercetin is found in many foods, such as apples, grapevines, berries, broccoli, red onions, capers and tea. It has anti-inflammatory, anti-infectious, antioxidant, anticancer/ chemo-preventive, neuro-protective,

antihypertensive, and blood glucose-lowering properties (Fürst and Zündorf, 2014). There are some other plants which are specifically used against OA such as *Achillea fragrantissima*, *Capparis spinose*, *Citrus colocynthis*, *Laurus nobilis*, *Ruta chalepensis*, *Urtica pilulifera*, etc (Ali-shtayeh, 2000). There are some other drugs which are under trial or not yet revealed as OA drugs (resveratrol, piceatannol, quercetin). This interest in alternative medical practices is growing fast but requires a more thorough investigation into their safety and efficacy (Soeken *et al.*, 2003).

1.4.3.1 Resveratrol

3,5,4-trihydroxy-trans-stilbene (resveratrol), a naturally occurring polyhydroxylated stilbene, is widely present in grapes, red wine, mulberries, peanuts and other edible plants. Resveratrol was first isolated in 1940 as a constituent of the roots of white hellebore (*Veratrum grandiflorum* O. Loes) (Aggarwal *et al.*, 2004). Resveratrol may be classified either as a polyphenol or as a phytoestrogen and has the stilbene core structure. This naturally occurring stilbene exerts protective effects against experimentally induced carcinogenesis (Jang *et al.*, 1997). However, the molecular mechanisms underlying the chemo-preventive activity of resveratrol are still unclear. Resveratrol also has substantial anti-inflammatory and antioxidant activity (Richardson *et al.*, 2000). The compound has anticancer, apoptosis properties (Dong, 2003), gives

cardiovascular protection (Bradamante *et al.*, 2006) and neuroprotection (Virgili and Contestabile, 2000). It has been reported that resveratrol induces p53-dependent apoptosis in several different cancer cell lines (Tang *et al.*, 2006), including cells derived from cancer of the prostate (Lin *et al.*, 2002; Shih *et al.*, 2004), thyroid (Shih *et al.*, 2002), and breast (Zhang *et al.*, 2004). Resveratrol is a non-selective inhibitor of COX-1 and COX-2 (Piotrowska *et al.*, 2012). It has been stated that resveratrol can suppress the activation of NF- κ B (Manna *et al.*, 2000) and downregulate inflammatory gene products such as COX-2, 5-LOX, IL-1b, and IL-6 (Manna *et al.*, 2000), all of which play a crucial role in arthritis.

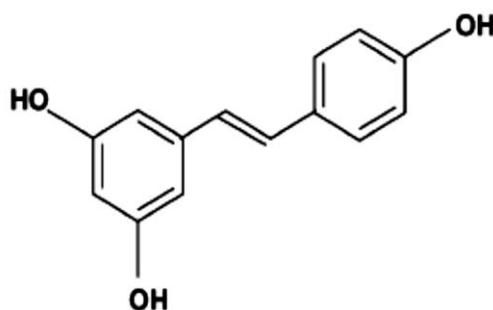


Figure 1.5: Molecular structure of resveratrol

1.4.3.2 Piceatannol

Piceatannol (3,3',4,5-tetrahydroxy-trans-stilbene/ 3-hydroxyresveratrol/ astringinine), a hydroxylated analogue of resveratrol, is a naturally occurring polyphenol present in red wine (Renaud and de Lorgeril, 1992). Piceatannol has been classified as

a phytoalexin (Hung *et al.*, 2001, 2000; Wolter *et al.*, 2002) because it is synthesized in plants in response to fungal infection or other environmental stresses. The seeds of *Euphorbia lagascae* (Ferrini) were the first source used for the isolation of piceatannol. It was also suggested that the presence of a specific enzyme (cytochrome P4501B1 in humans) is involved in the metabolism of resveratrol to piceatannol (Piver *et al.*, 2004; Potter *et al.*, 2002). Piceatannol is an active ingredient in many plant sources, including passion fruit (*Passiflora edulis*), *Cassia marginata*, *Melaleuca leucadendron* (white tea tree), *Cassia garretiana* (Asian legume), *Rheum undulatum* (Korean rhubarb) (Matsuda *et al.*, 2000, 2001), *Euphorbia lagascae*, *Mezoneuron cucullatum*, *Vitis amurensis*, *Caragana tibetica*, *Rheum rhaponticum*, *Parthenocissus tricuspidata*, *Aiphanes aculeata*, *Arachis hypogaea*, *Vitis thunbergii*, *Ampelopsis brevipedunculata*, and *Vaccinium* berries (Piotrowska *et al.*, 2012). Piceatannol has been reported to possess anti-inflammatory properties and can significantly reduce lipopolysaccharides (LPS)-induced production of nitric oxide (NO) in macrophages (Djoko *et al.*, 2007; Islam *et al.*, 2004) or microglia (Youn *et al.*, 2009). The LPS-induced expression of inducible NO synthase (iNOS) can also be suppressed by piceatannol (Islam *et al.*, 2004; Jin *et al.*, 2006; Matsuda *et al.*, 2000). Like resveratrol, piceatannol has been reported to have an anti-tumorigenic potential (Waffo-Tégou *et al.*, 2001). Piceatannol possesses higher biological activity compared to resveratrol as an

inhibitor of cyclooxygenase-2 (COX-2) (Potter *et al.*, 2002). It has been reported that piceatannol provides a wide variety of preventive and therapeutic options against cancer (Jeong *et al.*, 2015). It could potentially be used to treat various cancers such as bladder cancer, prostate, and colorectal cancers and melanoma (Chang *et al.*, 2006; Wolter *et al.*, 2002; Larrosa *et al.*, 2004; Lee *et al.*, 2009). It serves as an antioxidant, anti-leukemic, anti-inflammatory agent (Kukreja *et al.*, 2013). It may prevent type 2 diabetes (Minakawa *et al.*, 2012), treat cardiovascular disease (Honma *et al.*, 2003), lead to the formation of bone (Chang *et al.*, 2006), and possesses anti-melanogenic activity (Matsui *et al.*, 2010). Piceatannol exerts its effect on multiple target sites including phosphoinositide 3-kinase (PI3K), spleen tyrosine kinase (Syk), COX2, and ATPase (Seyed *et al.*, 2016). Piceatannol appears to be a selective COX-2 inhibitor (Kutil *et al.*, 2014).

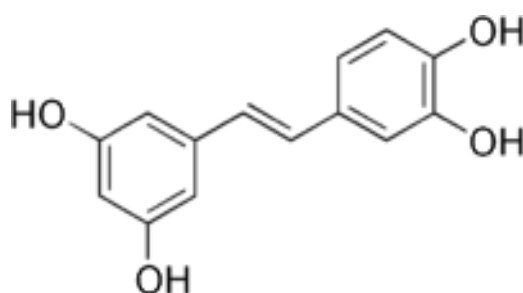


Figure 1.6: Molecular structures of piceatannol

1.4.3.3 Quercetin

Quercetin was first identified, isolated and described by Szent-Gyorgyi in 1936 (Harborne, 1986). Quercetin, the polyphenol, is characterized as a flavonol due to the presence of an oxygen-containing ring between 2 benzene rings (Figure 1.5). In food, however, quercetin is bound to mono or oligosaccharides via a glycosidic bond with the 3' hydroxyl group on the oxygen-containing ring (Miles *et al.*, 2014). Quercetin is available in naturally occurring foodstuffs e.g. onions (284-486 mg/kg), kale (110 mg/kg), french beans (32-45 mg/kg), broccoli (30 mg/kg), lettuce (14 mg/kg), tomatoes (8 mg/kg) (Hertog *et al.*, 1992). It is also found in beer, coffee, grape juice, chocolate milk, white wine and tea infusion at a concentration range of 1-25 mg/litre (Hertog *et al.*, 1993). Quercetin can act both on physiological and pathological conditions. It has properties such as; anti-oxidative (Tan *et al.*, 2009; Zhang *et al.*, 2008) and phyto-estrogenic (Piantelli *et al.*, 1995), and cardioprotective (Edwards *et al.*, 2007). It has been found to act as an immuno-suppressive agent by the inhibition of histamine release and inhibition of induction of cytotoxic T-cells (Fewtrell *et al.*, 1977; Schwartz *et al.*, 1982). Moreover, it has been observed to perform as an anti-obesity agent by decreasing triglycerides, total cholesterol, free fatty acids, insulin, and, at higher doses, decreasing body weight in obese Zucker rats (Rivera *et al.*, 2008). It has been

found as an anti-diabetic agent as it can protect β -cell function and viability against oxidative damage in insulin-secreting cell line INS-1 (Youl *et al.*, 2010). Quercetin has been observed as an anti-cancer agent (breast cancer/ MCF-7 cell line, fibrosarcoma/ WN113 cells, melanoma cell lines/ DB-1 [p53 wildtype] and S-Mel-p53 mutant, lung cancer stem-like cells/ A549 cell line, human ovarian cancer cell line/ A2780 and A2780)(Scambia *et al.*, 1994; Sliutz *et al.*, 1996; Hsu *et al.*, 2011; Nessa *et al.*, 2011; Thangasamy *et al.*, 2010). In mast cells, quercetin decreased the gene expression and production of tumor necrosis factor $\text{TNF}\alpha$, interleukin (IL)-1 β , IL-6, and IL-8 and also attenuated activation of NF- κ B and p38 mitogen-activated protein kinase (Min *et al.*, 2007).

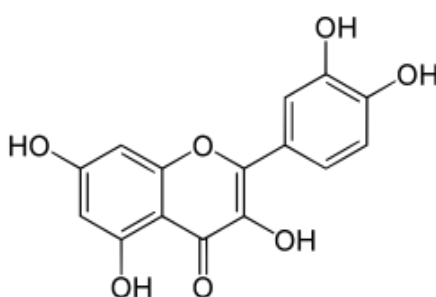


Figure 1.7: Molecular structure of quercetin

1.5 Pre-clinical studies

1.5.1 *In-vitro* studies

1.5.1.1 Cartilage explant cultures

Articular cartilage is a relatively acellular tissue which is comprised of 1-2% chondrocytes of the biomass which are responsible for the synthesis and maintenance of the extracellular matrix (Buckwalter and Mankin, 1997). Many *in vitro* culture models have been developed for the investigation of chondrocyte and cartilage biology, including explant models, several forms of three-dimensional culture systems, and monolayer cultures. In the cartilage explant model chondrocytes are maintained in their own matrix. Although chondrocytes in cartilage explants preserve their phenotypic stability, they have lower basal expressions of matrix proteins than are found *in vivo* (Stewart *et al.*, 2000). Explant models are often needed to study the synthesis and turnover of ECM molecules (Campbell *et al.*, 1989; Carney *et al.*, 1985) and can be used for different purposes such as studying of arthritic and non-arthritic cartilage from comparative studies on matrix composition and change (Malemud *et al.*, 1995). Articular cartilage explants can be maintained at steady-state for several weeks (Stewart *et al.*, 2000). This type of culture system is useful to examine how newly synthesized radiolabelled molecules are incorporated into the

existing cartilage matrix and also how they are turned over. A disadvantage of the explant culture system is that the newly synthesized aggregating PG (aggrecan) molecules may be found in high yield only by using solvents, which cause the dissociation of the large molecular mass aggregates (Campbell *et al.*, 1984).

1.5.1.2 Chondrocyte cultures

Chondrocytes, highly specialized cells of mesenchymal origin, are responsible for producing, maintaining and degrading the proteoglycan present in cartilage extracellular matrix (ECM) (Aydelotte and Kuettner, 1988). Chondrocytes synthesize a number of small proteoglycans and other specific and nonspecific matrix proteins (Maroudas *et al.*, 1992). It has also been observed that chondrocytes from deep zones synthesized significantly more proteoglycans than cells from the superficial zone (Aydelotte *et al.*, 1988). Moreover, it has been shown by Otero *et al.*, (2005) that the human adult articular chondrocyte reaches a fully differentiated state as an endpoint of development. Chondrocyte cultures are one of the most helpful methods to investigate intracellular and molecular phenomena associated with chondrocyte differentiation and activation (Goldring, 2000; Goldring and Berenbaum, 1999; Stokes *et al.*, 2001; Thirion and Berenbaum, 2004). Chondrocyte cell lines cannot fully substitute primary chondrocytes as there are

marked differences between the two phenotypes (Finger *et al.*, 2003; Loeser *et al.*, 2000). It is really difficult to acquire primary human chondrocytes in sufficient numbers from a single joint in order to perform an extensive study (Aigner *et al.*, 2001). After isolation, chondrocytes instantly lose their normal proliferative properties on a large scale. Even if chondrocytes start proliferation they show the loss of a differentiated phenotype, which includes not only a change in cell shape but also a very significant change in the pattern of gene expression (Stokes *et al.*, 2002). Thus experiments with these cells show high variability due to the different chondrocyte preparations.

1.5.2 *In-vivo* studies

In vivo models may provide the most precise reflection of the naturally-occurring whole joint disease (Madden *et al.*, 2012). *In vivo* studies help to understand the impact on the mechanical environment of articular cartilage and the mechanobiology of the tissue (Kersting *et al.*, 2005; Sweet *et al.*, 1992). Smaller animal models are chosen for preliminary screening as large species require higher maintenance costs and larger drug requirement. The mouse is the primary species for the generation of transgenic animals, including animals with gene deletions and gene over-expression. Despite these limitations, the prospective for identifying key enzymes involved in OA using transgenic animals make the

evaluation of OA models in mice the method of choice. To understand OA progression, using a microsurgical technique to produce instability in the knee joints is a common method. A common model involves destabilization of the medial meniscus (DMM) by transection of meniscotibial ligament anchoring the medial meniscus to the tibial plateau (Glasson *et al.*, 2007). Other commonly used microsurgical techniques involve a medial model by resection of the medial meniscus and medial collateral ligament (Kawaguchi, 2008), anterior cruciate ligament transection (ACLT) (Clements *et al.*, 2003). Another well-defined and recognised preclinical model of OA is the injection of monosodium iodoacetate (MIA) into the intra-articular space of the knee (Chandran *et al.*, 2009; Ivanavicius *et al.*, 2007; Pomonis *et al.*, 2005; Vonsy *et al.*, 2008). Intra-articular MIA causes transient inflammation followed by steady joint destruction with clinical OA (Pomonis *et al.*, 2005; Vonsy *et al.*, 2008). Whether this model of OA produces non-evoked or “ongoing” pain is not known (Liu *et al.*, 2011). It has been observed that animal models that spontaneously develop OA are likely to be more translatable for studying age-related human OA onset (Macfadyen *et al.*, 2019). The Dunkin Hartley guinea pig is a remarkable example in this regard (Jimenez *et al.*, 1997) which develops lesions resembling those of human OA and is used to study the etio-pathogenesis of the disease (Andrews *et al.*, 2012). These animals develop OA without surgical, chemical or environmental

manipulation (Jimenez *et al.*, 1997; Tonge *et al.*, 2013). However, the major drawback with these models is the time required for OA development, which can make studies prohibitively expensive and ultimately unfeasible (Macfadyen *et al.*, 2019). There are many other animal models which mimic key features of human OA in terms of natural history, mechanisms, signs and symptoms. However, one animal model does not completely represent all the signs and symptoms of human OA. It is advantageous to use models of OA with rapid progression for the purpose of drug development. This methodology may not be asking too much of the treatment as models of rapid onset of inflammation and joint destruction for inflammatory arthritis (e.g., collagen induced arthritis) have proven of value in disease modifying drug development for inflammatory arthritis. In contrast, a slower emerging OA model, more like human OA, may allow greater opportunities to control joint damage with a less potent drug (Poole *et al.*, 2010).

1.6 Aim and hypotheses

The aim of this thesis was to investigate the anti-inflammatory and dose-response effects of resveratrol, piceatannol and quercetin on an IL-1 β stimulated explant cartilage model in comparison with an NSAID (phenylbutazone), corticosteroid (prednisolone) and nitric oxide inhibitor (ethylisothiourea).

It was hypothesised that the effects of resveratrol, piceatannol and quercetin will follow a dose-response and they will be more potent than a NSAID (phenylbutazone) and corticosteroid (prednisolone) in reducing inflammatory markers of OA. Moreover they will play an effective role as a chondroprotective agent and have the potential to be disease modifying osteoarthritis drugs (DMOADs).

Chapter 2

General materials and methods

2.1 Collection of horse legs

Twelve pairs of legs were collected for each experiment from horses that had been euthanized at a licensed commercial abattoir in accordance with the 2015 animals slaughter act, UK (F Drury & Sons Ltd., Swindon). An inclusion criterion was that the horses were under ten years of age (as determined by the age recorded on their passport). Pairs of legs were transported back to the laboratory in sealed, labelled plastic bags. Gross decontamination was performed on the day of collection by cleaning with Teepol multi-purpose detergent (Teepol, Murray Rd, Orpington UK). The hooves were covered up with waterproof bags after cleaning. The legs were sealed in clean labelled plastic bags and stored in a cold room overnight.

2.2 Harvesting of cartilage

Twelve pairs of legs were collected for each experiment from horses that had been euthanized at a licensed commercial abattoir in accordance with the 2015 animals slaughter act, UK (F Drury & Sons Ltd., Swindon). An inclusion criterion was that the horses were under ten years of age (as determined by the age recorded on their passport). Pairs of legs were transported back to the laboratory in sealed, labelled plastic bags. Gross decontamination was performed

on the day of collection by cleaning with Teepol multi-purpose detergent (Teepol, Murray Rd, Orpington UK). The hooves were covered up with waterproof bags after cleaning. The legs were sealed in clean labelled plastic bags and stored in a cold room overnight.

The dorsal aspect of the metacarpophalangeal (fetlock) joint was clipped, and then scrubbed with Distel/Trigene (10% v/v) (Distel, high-level medical surface disinfectant, Scientific Laboratory Supplies, Tristel Solutions Limited, Lynx Business Park, Cambs, UK). A surgical scrub was performed over the incision site with chlorhexidine (Hibiscrub, Mölnlycke Health Care Ltd, DunsTable, Bedfordshire, UK) for a minimum contact time of 5 minutes, followed by application of 70% ethanol. The skin was incised on the dorsal aspect (front) of the metacarpophalangeal joint with a sterile scalpel using an S-shaped incision which extended on the medial and lateral aspect to the collateral ligaments. 70% ethanol was applied again over the subcutaneous tissue and the extensor tendons overlying the joint were transected. The scalpel blade used for the skin incision was discarded and a new sterile blade used to open the joint. The metacarpophalangeal joint was opened along the dorsal aspect, with the incision extending laterally and medially through the collateral ligaments and the sesamoid suspensory ligaments until the joint surfaces were fully exposed.

The cartilage was scored using the Osteoarthritis Research Society International (OARSI) macroscopic scoring system (McIlwraith *et al.*, 2010). An inclusion criterion for the study was a maximum score of 1 or 0 (Table 2.1) (Fig. 2.1 a-c). A scalpel was used to take full-thickness shavings of articular cartilage. Shavings were placed in 50 ml collection media consisting of 48 ml Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies™, Paisley, UK) with high glucose (1g/L), 110 mg/L sodium pyruvate, no phenol red, 2 ml antibiotic (streptomycin 5000 units/ml penicillin and 5 mg/ml streptomycin solution, Sigma Aldrich, Israel) and 4 mM L-glutamine (Sigma Aldrich, St. Louis, MO, USA).

2.1 Cartilage explant culture

After collection, cartilage shavings were transferred into washing medium for 20 minutes (sterile PBS + 10% antibiotic) and 3 mm cartilage disks were then produced using a sterile biopsy punch (Integra Miltex, Davis Drive, York, PA, USA). One hundred sixty-five cartilage disks (treatments included for histology also) were produced per horse, where three cartilage disks were added to each well of a 24-well plate (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG) with 1 ml of culture medium (CM). 100 ml of culture medium consisted of 98 ml of DMEM with high glucose (1g/L), 110 mg/L sodium pyruvate, no

Table 2.1: Macroscopic grading system to assess equine articular cartilage of the third metacarpal condyle (from McIlwraith *et al.*, 2010)

Overall macroscopic change		
Lesion	Grade	Description
Wear lines	0	None
	1	1 or 2 partial-thickness wear lines/joint surface
	2	3-5 partial-thickness or 1-2 full-thickness wear lines/joint surface
	3	>5 partial-thickness or >2 full-thickness wear lines/joint surface
Erosions	0	None
	1	Partial-thickness erosion, <5 mm in diameter
	2	Partial-thickness erosion, >5 mm in diameter
	3	Full-thickness erosion
Palmar arthrosis (osteochondral lesions distal palmar aspect of metacarpus)	0	None
	1	Partial-thickness erosion, <5 mm in diameter
	2	Partial-thickness erosion, purple discoloration, >5 mm in diameter
	3	Full-thickness erosion, purple discolouration, >5 mm in diameter

phenol red, 2 ml antibiotic (streptomycin 5000 units/ml penicillin and 5 mg/ml streptomycin solution) and 4 mM L-glutamine. Cartilage disks were then incubated overnight at 37°C and in 5% CO₂ (Figure 2.2). After overnight incubation, the medium was removed from each well and replaced with 600 µl of medium containing different experimental drug treatments or controls. Each treatment was carried out in triplicate, therefore n=1 is the average measurement over the three wells for each treatment, with one

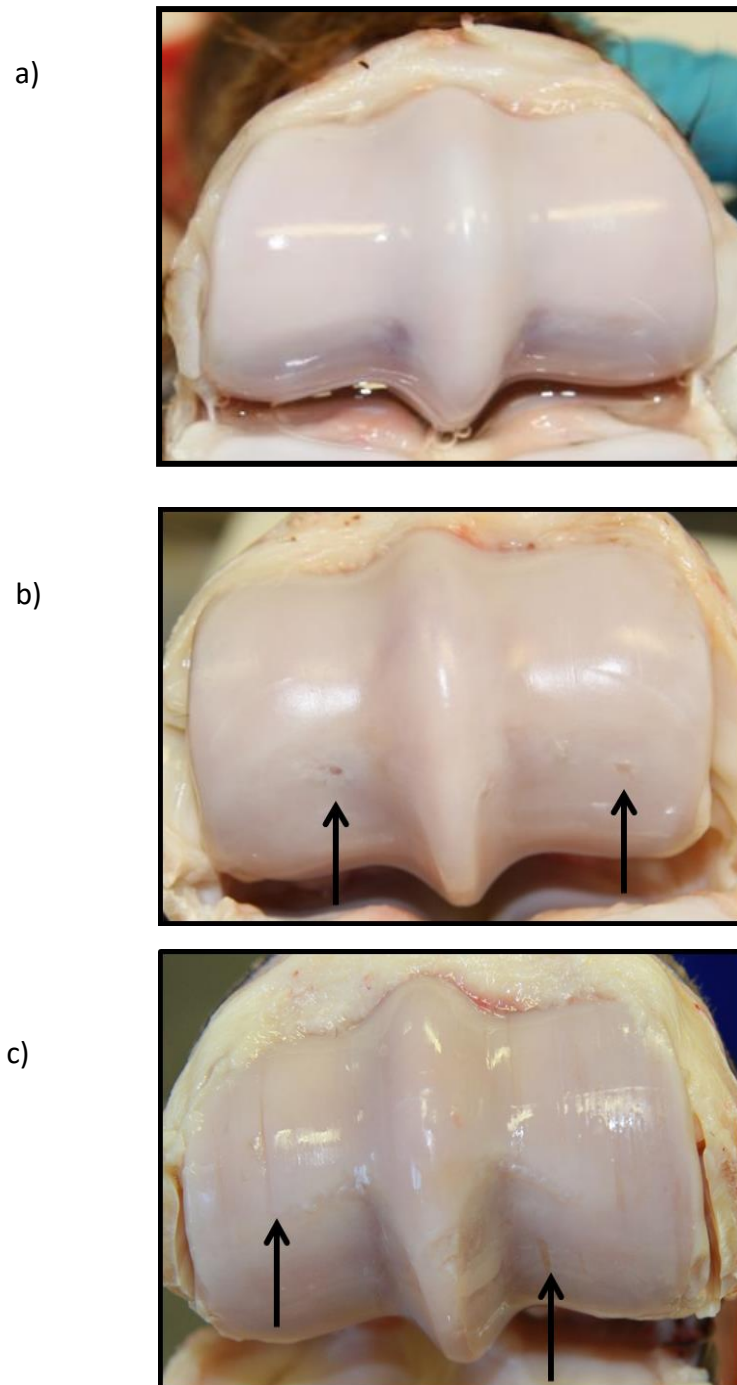


Figure 2.1(a-c): The gross image of a metacarpophalangeal joint (MCP) joint. (a) had a macroscopic score of 0 (no abnormalities) and was therefore considered suitable for this study; (b) the gross image of an MCP joint with a grade 2 erosion (>5mm erosion indicated by the black arrows), and therefore did not meet the inclusion criterion; and (c) the gross image of an MCP joint with grade 2 wear lines (two full-thickness wear lines illustrated by the black arrows), and therefore did not meet the inclusion criterion.

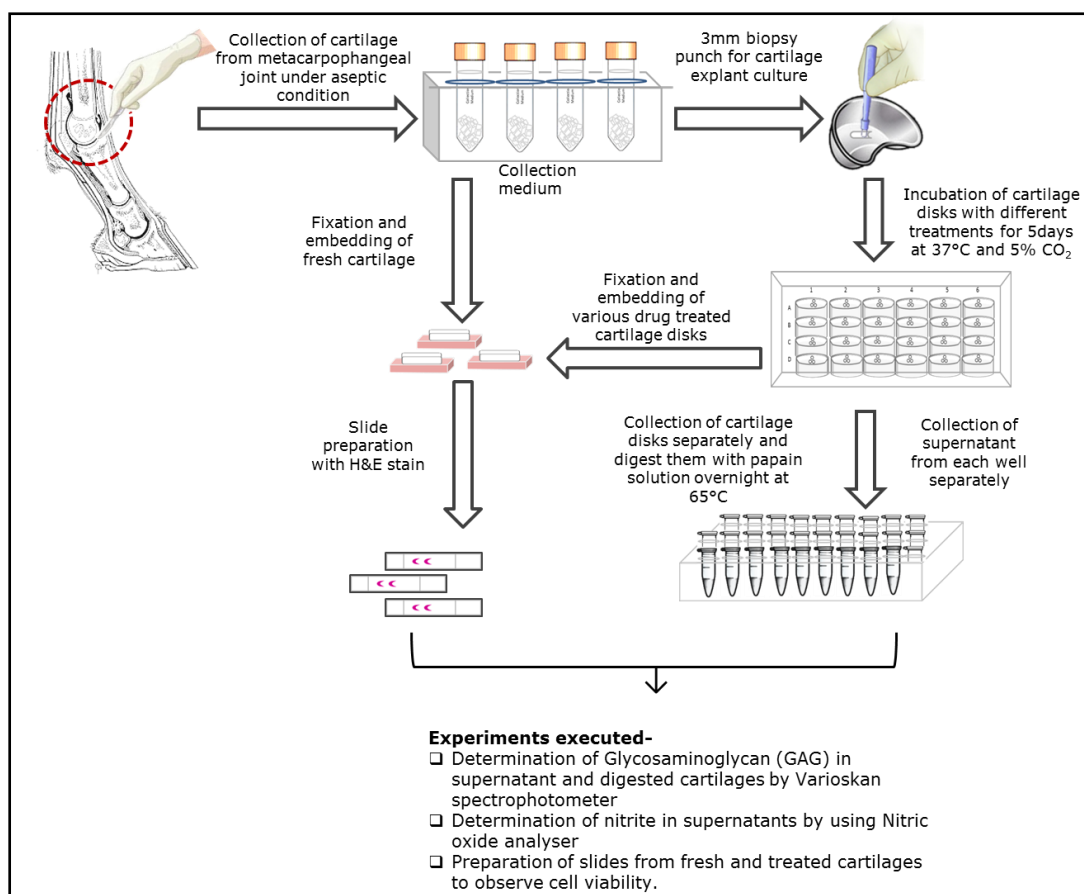


Figure 2.2: Schematic representation of the experimental procedure to set up and process cartilage explant culture treatments.

experiment being on cartilage extracted from a pair of legs from the same horse. All drug stocks were prepared with dimethyl sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK) at a concentration of 10 mg/ml. Drug stock solutions were then diluted 1:100 in culture medium leading to a final concentration of 100 µg/ml in each well. Therefore control wells contained culture medium only (600 µl culture medium), vehicle control wells and treatment wells contained culture media with 1% DMSO (v/v) (i.e. 6 µl of treatment prepared in DMSO plus 594 µl culture medium). Recombinant equine IL-1 β (R & D System, Minneapolis, USA) was prepared in PBS at a

concentration of 1 mg/ml for a final concentration of 10 ng/ml in the wells (i.e. 6 μ l of treatment prepared in IL-1 β plus 6 μ l DMSO plus 588 μ l culture medium).

After five days of culture, the supernatants from each well were collected and stored at -20°C. All treated cartilage disks from each well were digested separately overnight at 65°C in 600 μ l of 1.1 mg/ml papain solution. Only piceatannol (in the presence and absence of IL-1 β) treated cartilage disks were kept for digestion over a period of one week. Papain solution was prepared by dissolving 185 mg of papain from Papaya (Sigma Life Science, St. Louis, MO USA) in 175 ml of papain buffer (made up of 2.48 g dibasic sodium phosphate (Sigma Aldrich, St. Louis, MO USA) (97 mM), 158 mg cysteine hydrochloride (Sigma, Life Science, St. Louis, MO USA) (97 mM), 372 mg ethylenediamine tetraacetic acid disodium salt, dehydrate (EDTA) (Fisher Bioreagents, New Jersey, USA) (5.55 mM), and 180 ml RO water (GE Health Care, Life Science, Hyclone Laboratories, Logan, Utah). The pH of the buffer was adjusted to 6.5 using 1M hydrochloric acid (Acros organics, New Jersey, USA).

Treatments used for the detection of lipopolysaccharide (LPS) activity from the equine recombinant IL-1 β product in the absence/presence of DMSO were IL-1 β (IL), polymyxin B (PMB) (Alfa Aesar, Ward Hill, MA, USA) and LPS (Sigma Life Science, St. Louise,

MO, USA). Each treatment was carried out in triplicate. All drug stocks were prepared with phosphate buffered saline (PBS) (Gibco by Life Technologies, Grand Island, NY, USA). Three different experiments were executed to investigate if the equine recombinant IL-1 β was contaminated with LPS. LPS concentrations of 10 ng/ml (in the presence and absence of 1% DMSO in separate experiments) and 100 pg/ml (in the absence of 1% DMSO) were tested (plate design is shown in Appendix 1, Figure 1a).

In experiments investigating the effects of resveratrol and its analogue piceatannol using an explant cartilage culture model, prednisolone, phenylbutazone, S-ethylisothourea, resveratrol, and piceatannol (in the presence and absence of IL-1 β (10 ng/ml)) were compared with control, vehicle control (control + 1% DMSO) and IL-1 β (alone). All treatments used in this study were 100 μ g/ml. Plate design is shown in Appendix 1, Figure 1b.

In studies investigating the dose dependent effect of resveratrol and piceatannol, concentrations of 1 μ g/ml, 10 μ g/ml, 30 μ g/ml, 100 μ g/ml and 300 μ g/ml (in the presence and absence of IL-1 β (10 ng/ml)) were compared with control, vehicle control (control + 1% DMSO) and IL-1 β (alone). Plate design is shown in Appendix 1, Figure 1c.

In a comparative study investigating quercetin, prednisolone, phenylbutazone and S-ethylisothiourea, quercetin (high dose, 100 $\mu\text{g/ml}$ and low dose, 10 $\mu\text{g/ml}$) (in the presence and absence of IL-1 β (10 ng/ml)) were compared with control, vehicle control (control + 1% DMSO) and IL-1 β (alone). All treatments used in this study were 100 $\mu\text{g/ml}$ (except quercetin at low dose 10 $\mu\text{g/ml}$) (Appendix 1, Figure 1d). For the dose dependent effects of quercetin, concentrations of 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$ (in the presence and absence of IL-1 β , 10 ng/ml) were compared with control, vehicle control (control + 1% DMSO) and IL-1 β (alone). Plate design is shown in Appendix 1, Figure 1c.

2.2 Nitric oxide analysis

Two nitric oxide analyzers (Sievers 280 and Sievers 280i, Nitric Oxide Analyzer, GE Water and Process Technologies, Boulder, CO, USA) were used to detect nitrite levels within the supernatant as an indirect indicator of nitric oxide levels. A sodium nitrite standard (Fisher Scientific) was used to prepare a standard curve from 0 to 500 nM (1, 10, 50, 100, 300 and 500 nM). A 1:10 dilution of the supernatant with reverse osmosis (RO) water was prepared and nitrite concentration was determined. Each diluted sample was injected directly into the analyser and the concentration of nitrite within the sample was automatically calculated from the area under

the detected peak. Nitrite production in all treatments was compared with vehicle control and IL-1 β individually.

2.3 PGE₂ detection

A competitive ELISA (Prostaglandin E2 Assay, R&D systems, 19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK) was used to quantify PGE₂ concentrations in the supernatant. To remove particles which may interfere with the ELISA, supernatants were centrifuged at 664g for 10 minutes prior to analysis. Previous work had indicated dilution of supernatants was not necessary (Smith *et al.*, 2013). With the exception of the recommended dilution, the provided protocol by R & D System was followed. Absorbance was read at 450 nm and 630 nm using a Varioskan Flash microplate reader. GraphPad Prism 7 was used to interpolate concentrations of PGE₂ from the sigmoidal standard curve (4 parameters, log concentration).

2.4 Glycosaminoglycan (GAG) analysis

The amount of GAG was determined from both the supernatant and digested cartilage using a spectrophotometric assay. All reagents and samples used were pre-warmed in an incubator at 25°C for an hour. 1,9-dimethylmethylen blue (DMMB) (Sigma Life Science, St.

Louise, MO, USA) solution was used for spectrophotometer analysis and prepared using 0.05 mM DMMB, 0.03 M sodium formate (Sigma Life Science, St. Louise, MO, USA), 2 ml formic acid (Sigma Aldrich, St. Louise, MO, USA), 5 ml absolute ethanol (Fisher Scientific, Loughborough, UK) and 93 ml RO water. The pH was then adjusted to 3.5 with 1M HNO₃. The solution was wrapped with aluminium foil and placed in a cool dark place (as DMMB medium is light sensitive). In order to quantify GAG levels, chondroitin sulfate (Sigma) standards were prepared using RO water. Standards ranged from 0 to 300 µg/ml (0, 15, 30, 45, 60, 75, 100, 200 and 300 µg/ml).

To analyse GAG levels within the conditioned media, a 1:3 dilution of each supernatant was performed with RO water, whereas a 1:6 dilution of the digested cartilage was performed to analyze GAG levels in cartilage (the exception was quercetin experiments where 1:2 dilution of supernatant and 1:4 dilution of digested cartilage were used). After the dilutions, the assay protocol was the same for standards, supernatants, and digested cartilage.

40 µL of each standard and the diluted sample were added to the plate in duplicate (flat bottomed 96 well plates) (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG). 200 µL of DMMB solution was then added to each well. After adding DMMB, plates were wrapped with aluminium foil and kept in an

incubator at 25°C for 30 minutes. Plates were read immediately using a Varioskan Flash (Thermo Fisher, Scientific, Vantaa, Finland) microplate reader set at 540, 600 and 650 nm.

2.5 Histology

Fresh cartilage shavings were collected from each horse during cartilage extraction and cartilage disks (3 mm biopsy punch) were collected after five days explant culture. Both cartilage shavings and biopsy punches were then fixed in 10% (v/v) neutral buffered formalin (Leica) for 24-48 hours (Figures 2.2, 2.3 and 2.4) for histological assessment. Tissue was then processed, embedded in paraffin and transversely sectioned at five microns using an RM2255 rotary microtome (Leica). The slides were then stained with Haematoxylin & Eosin (H&E) or Safranin O and fast green (SOFG) following a modification of established protocols (Appendix 1 A-D).

In Chapter 3, histological observation of fresh, control, Polymyxin B, IL-1 β (in the presence and absence of Polymyxin B), LPS (in the presence and absence of Polymyxin B) cartilage was performed for SOFG staining to observe GAG breakdown. In Chapter 4, histological assessment of fresh, control, IL-1 β (alone), resveratrol (in the presence and absence of IL-1 β) and piceatannol (in the presence and absence of IL-1 β) treated cartilage was performed for H and E staining (for cell viability count) and SOFG staining (for GAG

breakdown). In Chapter 5, any dose dependent effect of resveratrol on cartilage was investigated by the histological study of fresh, control, IL-1 β (alone) and resveratrol treatment at 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 300 $\mu\text{g/ml}$ (in the presence and absence of IL-1 β), using SOFG staining to observe GAG degradation. In Chapter 6, the dose dependent effect of piceatannol was investigated in histological study of fresh, control, IL-1 β (alone) and piceatannol treatment at 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$, 300 $\mu\text{g/ml}$ (in the presence and absence of IL-1 β), using SOFG staining to observe GAG degradation. In Chapter 7, histological observation of fresh cartilage, control, IL-1 β treated (alone) and quercetin treated (low or high dose in the presence and absence of IL-1 β) was performed to observe GAG degradation. For the dose dependent effect of quercetin; fresh, control, IL-1 β (alone), 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 30 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$ quercetin (in the presence and absence of IL-1 β), were used for SOFG staining to observe GAG degradation.

2.6 Cell viability count

Three different sections (minimum interval between each section was ≥ 20 micron) from each treatment was observed under 40x magnification. Dead cells were determined by pyknotic nuclei or

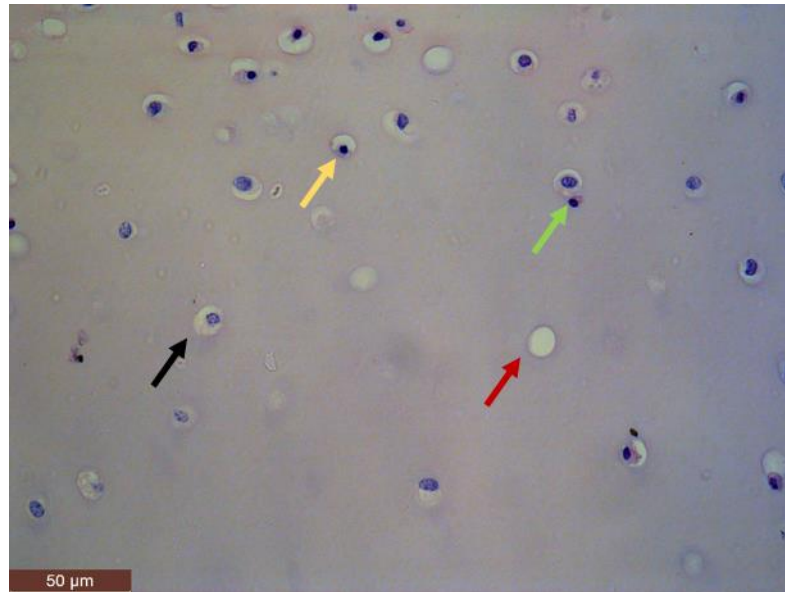


Figure 2.3: Representation of the microscopic image of cartilage explant after five days culture. Black arrow indicates a live cell, the red arrow is showing a dead cell (empty lacunae), green arrow is showing nucleus in extracellular matrix and yellow arrow is showing dead cells (pyknotic nucleus). Cells were observed under 40x magnification.

empty lacunae; whereas live cells were determined by the distinct nucleus and cell membrane (Figure 2.3). Percentage of live cells and dead cells were then calculated (with significance) using Microsoft Excel 2010 and GraphPad Prism 7.

2.6.1 GAG analysis

GAG degradation was observed by SOFG stain uptake by cartilage after preservation and tissue processing. In Figure 2.4, the blue colour represents degraded GAG whereas red colour indicates intact GAG. The scoring was performed based on the ratio of red to blue

colour uptake by the cartilage section following OARSI standards (Table 2.2) (Kraus *et al.*, 2010).

Table 2.2: Microscopic grading system to assess GAG degradation in equine articular cartilage by SOFG stain uptake (Kraus *et al.*, 2010)

Overall microscopic change		
SOFG stain uptake	0	Normal staining
	1	Less than 25% loss of staining characteristics
	2	25-50% loss of staining characteristics
	3	50-75% loss of staining characteristics
	4	Greater than 75% loss of staining characteristics

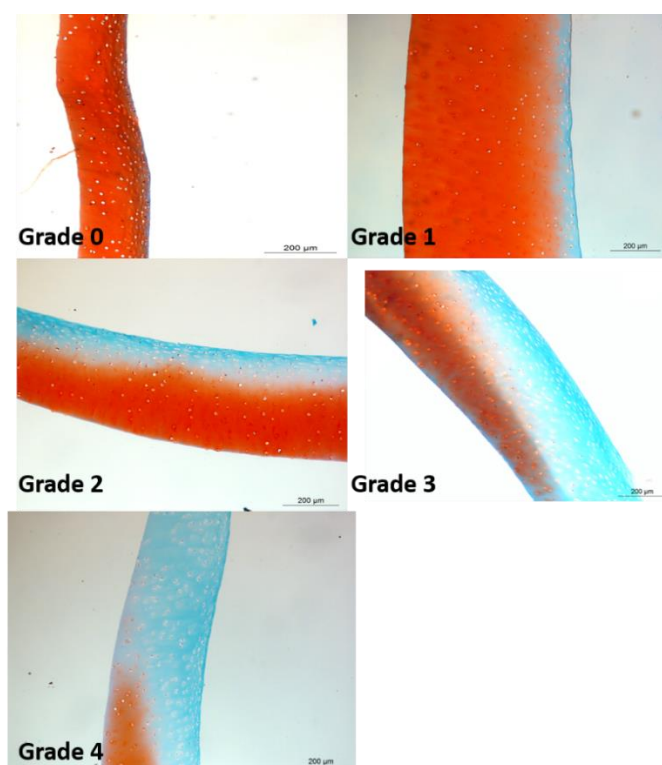


Figure 2.4: Representative examples of the different grades observed in SOFG staining (grade 0-4) following OARSI standards.

In all studies, GAG degradation by SOFG staining techniques for each treatment was observed from wells selected randomly from two different horses/experiment (one well from one horse/experiment hence $n=2$).

2.7 Data analysis

2.7.1 Statistical analysis

2.7.2 Dose-response curves for piceatannol and resveratrol

Data obtained from experiments on nitric oxide and PGE₂ production, cell viability count and GAG degradation were analysed using Microsoft Excel 2010 and GraphPad Prism 7. One-way analysis of variance (ANOVA) was used to compare mean data between all treatments. A post-hoc Dunnett test (non-paired) was performed to determine any statistical difference based on P-values between treatments and either vehicle control or IL-1 β only. Non-paired tests were used, as while treatment comparisons were in the same horse, cartilage from different positions of pooled joints were used.

Dose-response curves were produced for the GAG and nitric oxide measurements. All dose-response curves were generated using GraphPad Prism 7. For dose-response in GAG, percentage release values were used whereas detected nitrite values were used for nitric

oxide dose-response curve. The percentage of GAG release for each concentration of piceatannol/ resveratrol/ quercetin in the absence of IL-1 β was subtracted from the corresponding concentration's percentage of GAG release in the presence of IL-1 β . The value for vehicle control was subtracted from the value for IL-1 β to normalize IL-1 β response. All adjusted readings were then divided by the normalized IL-1 β value to give a normalized percentage response. The same procedure was also applied for nitric oxide production. Graphs were then produced from this data and an IC₅₀ determined by using GraphPad Prism7. For NO and IL-1 β stimulated GAG, the log (inhibitor) vs. normalised response – variable slope function (4 parameters) was used to generate dose-response curves. A restricted curve was used such that negative and greater than 100% values can be overlooked. From this curve, LogIC₅₀, standard error of LogIC₅₀ and IC₅₀ values were determined.

Chapter 3

Validation of inflammatory stimulant and vehicle in equine cartilage explant model

3.1 Introduction

In cartilage explant culture, chondrocyte biology can be investigated in an organized structure of cartilaginous skeletal elements (Manuela and Andrea, 2014). Therefore, the morphological and biosynthetic characteristics of the chondrocytes will remain intact due to the controlled and physiologically relevant environment (Dumont *et al.*, 1999).

Bacterial lipopolysaccharides (LPS) can induce cartilage matrix degradation in live explant cultures by direct stimulation of protease and collagenase secretion by chondrocytes (Jasin, 1983). According to the literature, stimulation of the inflammatory pathway (GAG breakdown) by lipopolysaccharide may be due to activation of cells of the innate immune system (macrophages and neutrophils) which synthesize proinflammatory factors (IL-1 β and TNF, MMPs and free radicals) (Lorenz *et al.*, 2013). Polymyxin B is usually used as a specific inhibitor of lipopolysaccharide (Tsuzuki *et al.*, 2001).

Interleukin-1 β (IL-1 β) is an important pro-inflammatory cytokine involved in inflammatory and autoimmune diseases (Dinarello, 1998). It has been shown that in human peripheral mononuclear blood cell culture the secretion of IL-1 β occurs in two steps. In the first step, an inflammatory signal by LPS promotes the synthesis and

cytoplasmic accumulation of the inactive precursor (pro-IL-1 β) (Schindler & Dinarello, 2019). A second signal, exogenous ATP (Adenosine triphosphate), triggers caspase-1-mediated processing of pro-IL-1 β (Burns *et al.*, 2003).

In this study, the recombinant equine IL-1 β product was used at a concentration of 10 ng/ml throughout the experiments. This was based on a previous study which showed that IL-1 β at 10 ng/ml concentration was found to be the minimum dose to induce GAG breakdown in an equine explant cartilage culture (Clutterbuck *et al.*, 2009). However, there is a possibility that the recombinant IL-1 β product is contaminated with lipopolysaccharide as it is extracted from *E. coli* though the manufacturer claims that the endotoxin level is <1.0 EU per 1 μ g. In order to investigate any contribution of *E. coli*, to the IL-1 β recombinant product, two different concentrations (100 pg/mg and 10 ng/ml) of lipopolysaccharide were used in this study in the presence and absence of DMSO. The 10 ng/ml and 100 pg/ml lipopolysaccharide mimic 100% and 1% endotoxin (LPS) contamination in recombinant equine IL-1 β product respectively.

for a nutraceutical to exert its pharmacological effect in a biological system it needs to be dissolved in a solution. Dimethyl sulfoxide (DMSO) is an important solvent which can solubilize a wide variety

of poorly soluble polar and nonpolar molecules (Galvao et al., 2014) and it is commonly used as a solubilizing vehicle.

In this study, it was hypothesized that bacterial lipopolysaccharide will have a negligible contribution to inflammatory response exerted by the recombinant equine IL-1 β product. In addition, it was hypothesized that DMSO will be an appropriate vehicle which will not interfere with (a) the inflammatory response in equine cartilage explant culture and (b) anti-inflammatory properties of the treatments used in these cultures

3.2 Estimating LPS activity in equine recombinant IL-1 β product using a low dose (100 pg/ml) LPS in the absence of DMSO

3.2.1 Results

3.2.1.1 Gross morphology from cartilage explant culture

Equine cartilage disks were incubated with culture medium only (Control, CO), polymyxin B (PMB) alone (13 IU/ml), lipopolysaccharide (LPS) (100 pg/ml) or IL-1 β (IL) (10 ng/ml)

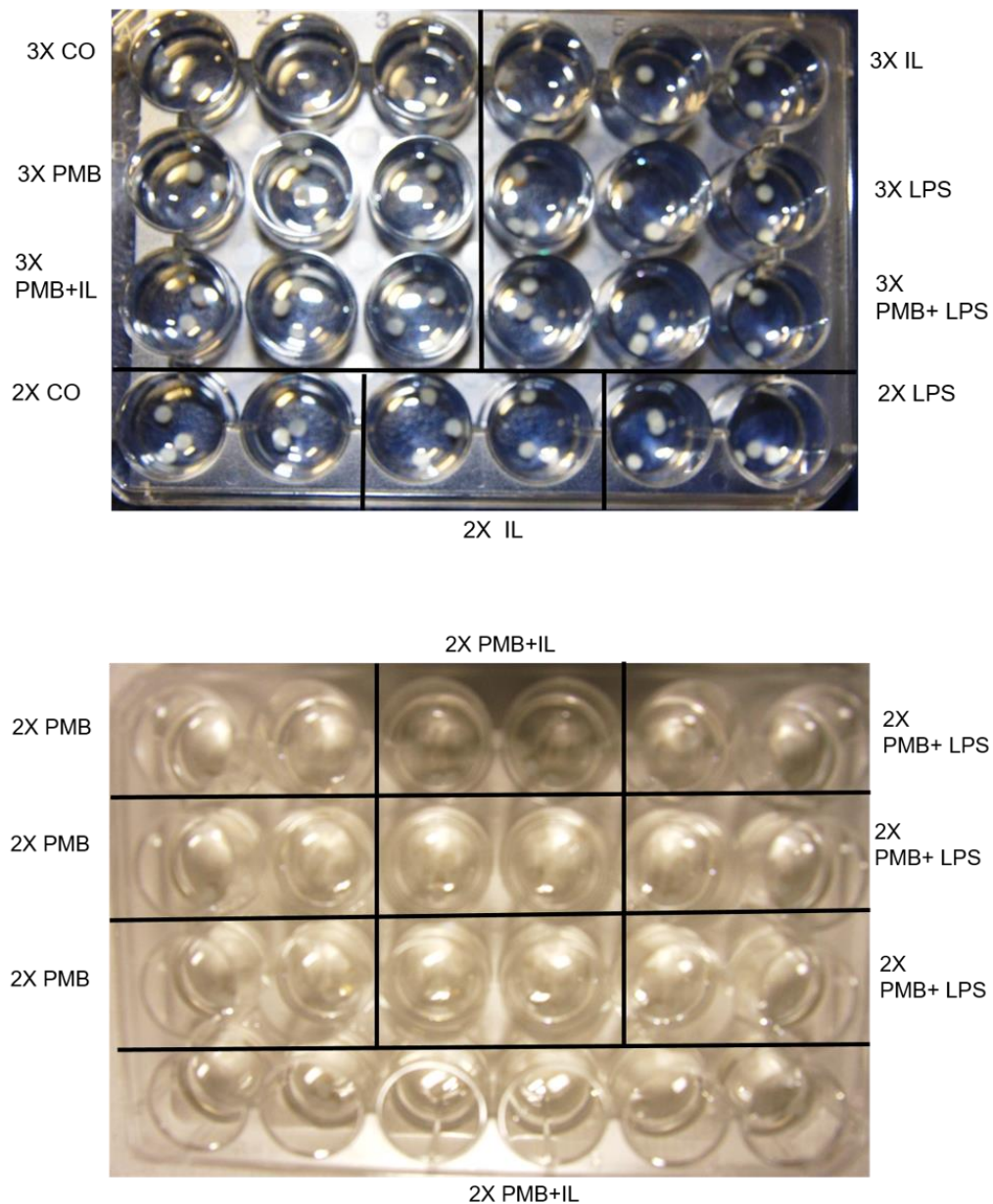


Figure 3.1: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), Polymyxin B (PMB) (13 IU/ml) or Lipopolysaccharide (LPS) (100 pg/ml) and interleukin-1 β (10 ng/ml, IL) in presence and absence of PMB.

(alone), or LPS or IL in the presence and absence of polymyxin B, all cartilage discs showed no visible gross changes during five days of culture (Figure 3.1).

3.2.1.2 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 2, Figure 1a-d). Nitrite production for all treatments, in both the presence and the absence of polymyxin B, were compared with control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 11.05, R^2 value 0.7542, one-way ANOVA) across the treatments. Nitrite production was the highest in IL-1 β (alone) treatment (Figure 3.2a, Table 3.1) and only IL-1 β (alone) (27.3 ± 11.1 μM) treatment showed a significant difference in nitrite production in comparison with control (6.86 ± 1.84 μM) (Figure 3.2a). Lipopolysaccharide (alone) did not induce nitrite production (Figure 3.2a). All treatments were significantly different in nitrite production when compared with IL-1 β alone (Figure 3.2a).

Table 3.1: Computed concentration (μM) of nitrite production in four horses.

	CO	IL	LPS	PMB	PMB+IL	PMB+LPS
HORSE 1	6.40	25.2	7.93	2.53	8.40	2.32
HORSE 2	5.70	43.1	6.73	4.19	14.6	7.42
HORSE 3	5.77	17	11.5	4.97	17.5	10.1
HORSE 4	9.57	23.9	9.23	3.93	16.8	2.17
MEAN	6.86	27.3	8.84	3.91	5.5	14.3
SD	1.84	11.1	2.03	1.02	3.91	4.13

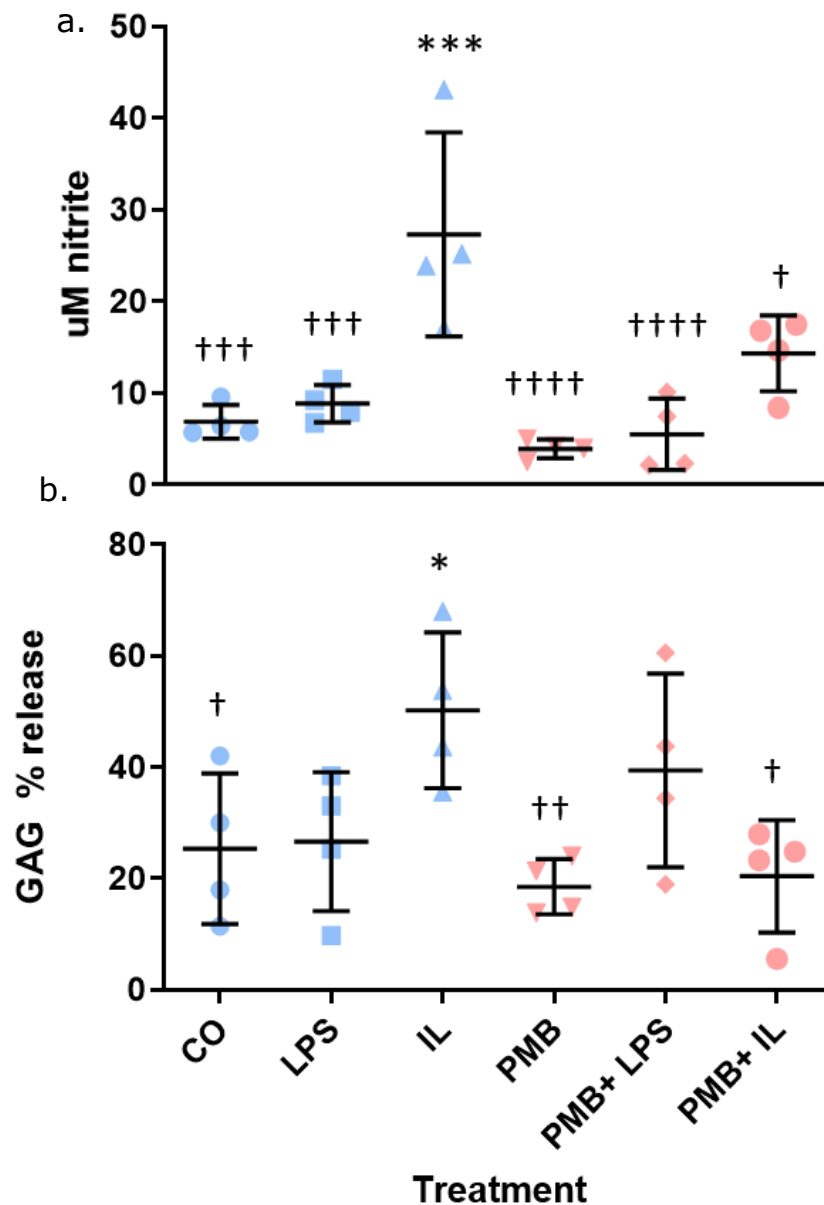


Figure 3.2: Interleukin-1 β (IL) and Lipopolysaccharide (LPS)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) and lipopolysaccharide (LPS) (100 pg/ml), IL -1 β (IL) (10 ng/ml) in presence and absence of polymyxin (PMB) (13 IU/ml) or PMB alone. Controls were culture media only (CO). Data are presented as scatter plots with mean and standard deviation. The release of (a) nitric oxide (nitrite) (n=4) and (b) glycosaminoglycan (GAG) (n=4) were measured and compared using one-way ANOVA with Dunnett's post-test (*P < 0.05 or ***P < 0.001, significant difference compared to control; +P < 0.05, ++P < 0.01, +++P < 0.001, significantly different to IL alone).

3.2.1.3 GAG analysis

3.2.1.3.1 Colorimetry

Calibration curves of GAG degradation for four horses are shown in Appendix 2 (Figure 2 a-d). Comparison of GAG % release showed there was a significant difference ($P=0.0166$, F value 3.759, R^2 value 0.5108, one-way ANOVA) across the treatments. All treatments except IL-1 β (alone) showed no significant difference in mean GAG production in comparison with control (25.4 ± 13.5 % release) (Figure 3.2b, Table 3.2) and IL-1 β (alone) (50.3 ± 14 % release) treated cartilage showed the highest GAG breakdown among all treatments. IL-1 β in the presence of polymyxin B showed similar GAG breakdown as control (Figure 3.2b). Lipopolysaccharide (alone) showed very low GAG breakdown which was similar to control (Figure 3.2b).

Table 3.2: Computed concertation (%) of GAG release in four horses.

	CO	LPS	IL	PMB	PMB +LPS	PMB+ IL
HORSE 1	11.4	33	43.6	13.8	19	24.8
HORSE 2	30	25.3	68.1	14.8	60.6	23.3
HORSE 3	18	38.5	35.6	24	34.4	28
HORSE 4	42	9.75	53.7	21.4	43.7	5.55
MEAN	25.4	26.6	50.3	18.5	39.4	20.4
SD	13.5	12.5	14	4.97	17.4	10.1

3.2.1.3.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4 (Figure 3.3 (a-g) according to OARSI. Under 10x magnification, qualitative data of GAG degradation determination is presented here (Figure 3.3 a-g). Fresh cartilage was graded as 1 (Figure 3.3 a), control, polymyxin B (alone), lipopolysaccharide (alone) and IL-1 β (in the presence of polymyxin B) treated cartilage were graded 1 (Figure 3.3 b, c, d, and g). IL-1 β alone treated cartilage was graded 4 (Figure 3.3 e) and lipopolysaccharide in the presence of polymyxin B treated cartilage was graded as 3 (Figure 3.3 f) according to OARSI.

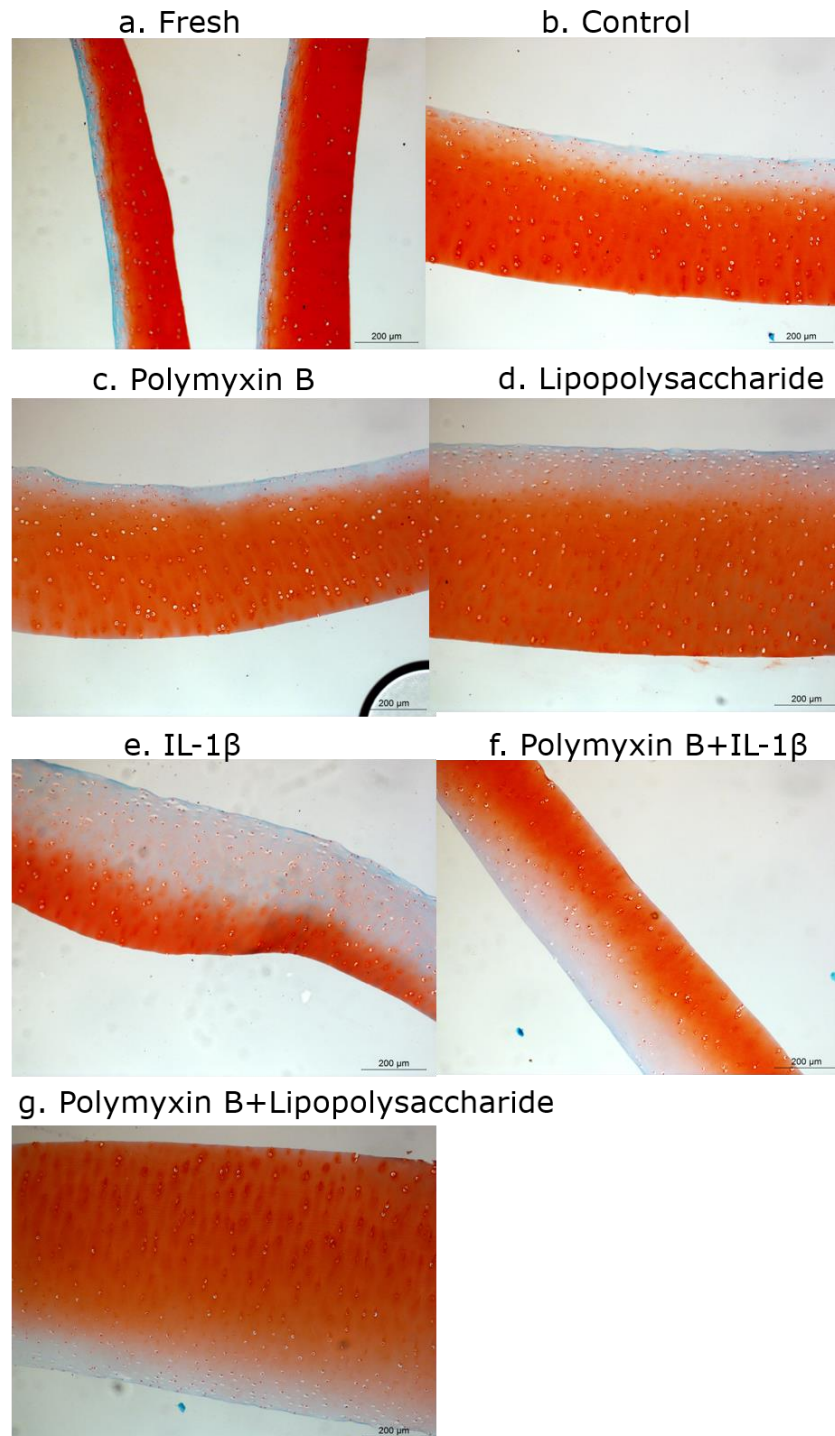


Figure 3.3: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh equine cartilage (a) and explant cultures (b-g). Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), polymyxin B (PMB) (13 IU/ml) (c), lipopolysaccharide (LPS) (100 pg/ml) (d), IL -1 β (IL) (10ng/ml) (e), PMB+ LPS (f), PMB + IL 1 β (g). Section a was graded as 1, sections b, c, d and g were graded as 2, sections e was graded as 4 and f was graded as 3 according to OARSI. Magnification x10; scale bars 200 μ m.

3.3 Estimating LPS activity in equine recombinant IL-1 β product using high dose (10 ng/ml) LPS in the absence of DMSO

3.3.1 Results

3.3.1.1 Gross morphology from cartilage explant culture

Equine cartilage disks were incubated with culture medium only (Control, CO), polymyxin B (PMB) alone (13 IU/ml), lipopolysaccharide (LPS) (10 ng/ml) or IL-1 β (IL) (10 ng/ml) (alone), or LPS or IL in the presence and absence of polymyxin B, all cartilage discs showed no visible gross changes during five days of culture (Figure 3.4).

3.3.1.2 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 2, Figure 3.5a). Nitrite production for all treatments, in both the presence and the absence of polymyxin B, were compared with control and IL-1 β individually (n=3). Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F

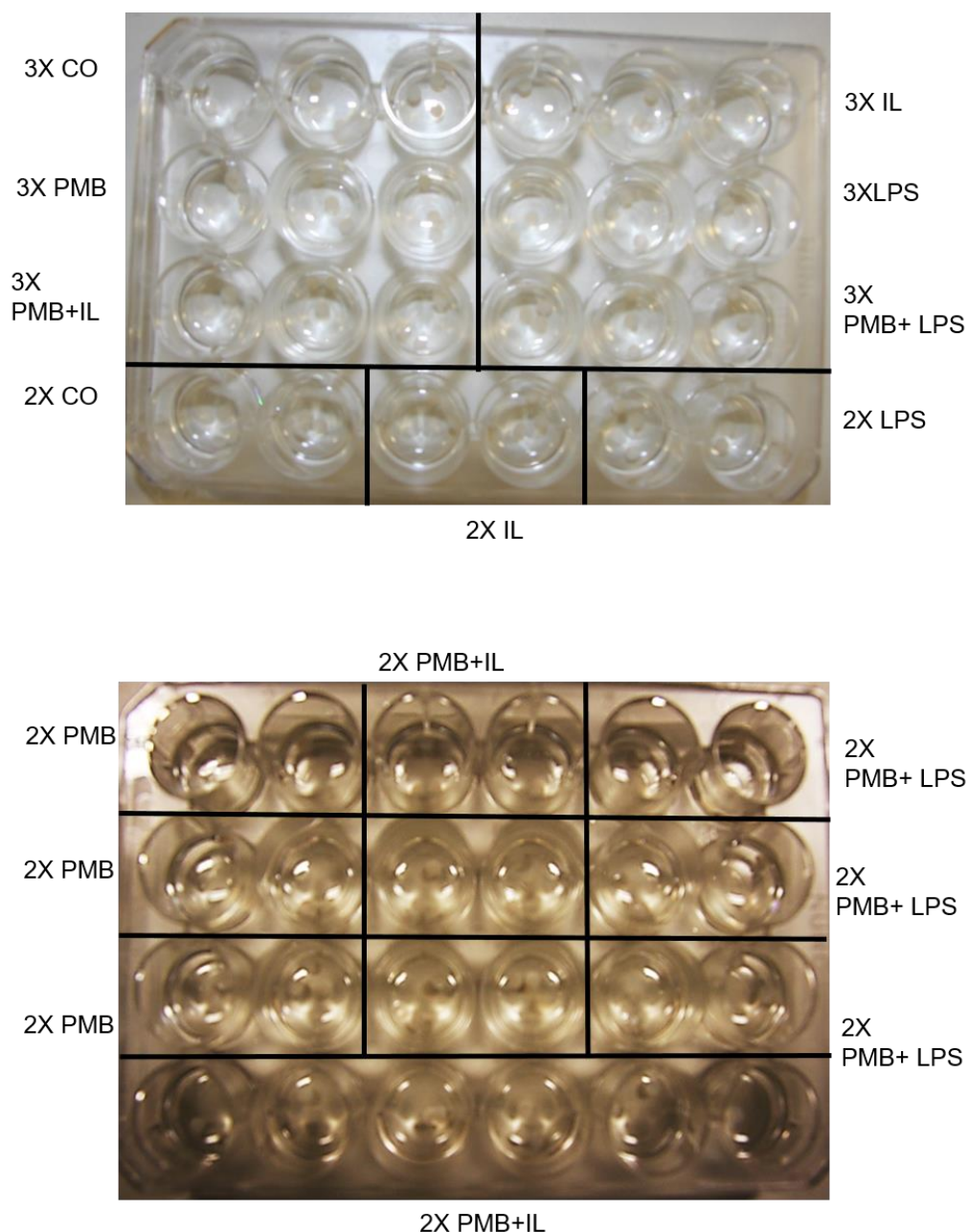


Figure 3.4: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), Polymyxin B (PMB) (13 IU/ml) or Lipopolysaccharide (LPS) (10 ng/ml) and interleukin-1 β (10 ng/ml, IL) in presence and absence of Polymyxin B (PMB).

value 38.67, R^2 value 0.9416, one-way ANOVA) across the treatments. Nitrite production in all treatments was compared

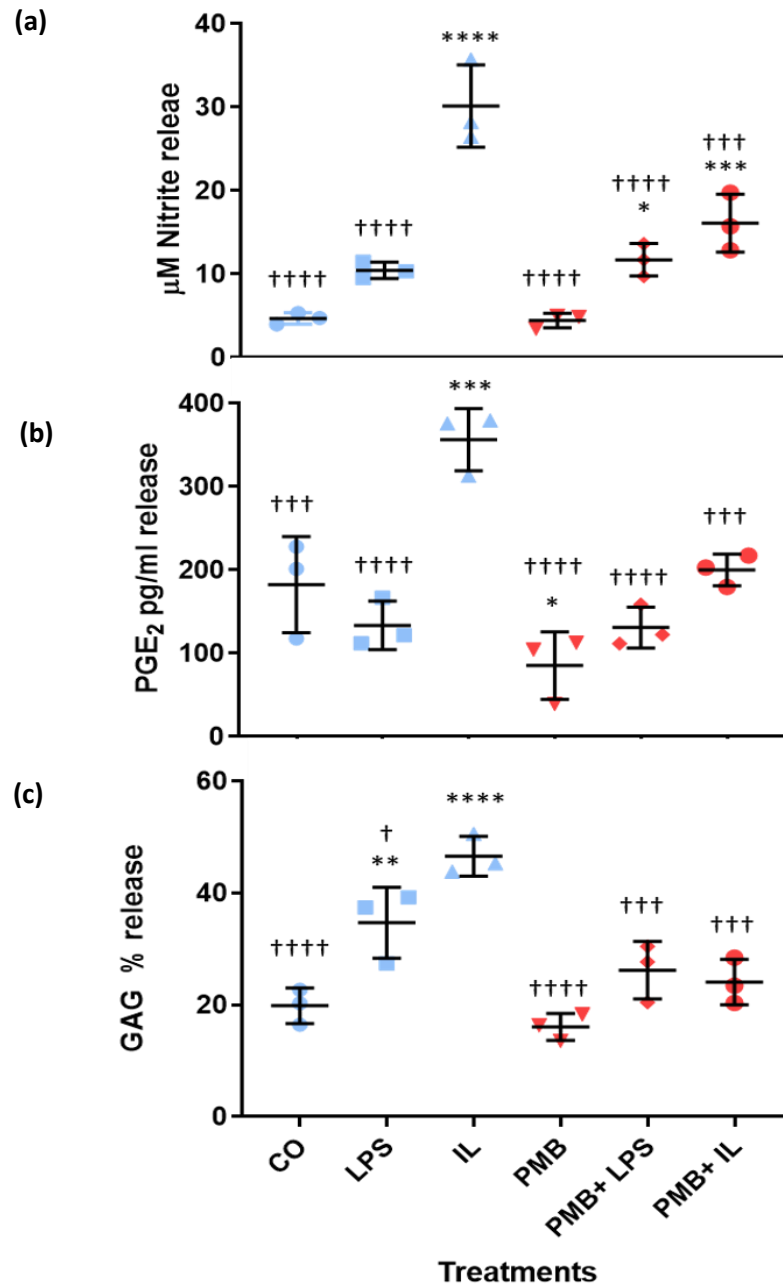


Figure 3.5: Interleukin-1 β (IL) and Lipopolysaccharide (LPS)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were stimulated for 5 days with lipopolysaccharide (LPS) (10 ng/ml), IL -1 β (IL) (10 ng/ml) in presence and absence of polymyxin B (PMB) (13 IU/ml) and PMB alone. Controls were culture media only (CO). Data are presented as scatter dot plot with mean and standard deviation (n=3). The release of nitric oxide (nitrite) (n=3) (a), prostaglandin (PGE₂) (n=3) (b), glycosaminoglycan (GAG) (n=3) (c) were measured. One-way ANOVA with Dunnett's post-test was performed. (*P < 0.05, **P < 0.01, ***P < 0.001 or ****P < 0.0001, significant difference compared to control; †P < 0.05, ††P < 0.01, †††P < 0.001 or ††††P < 0.0001, significantly different to IL alone).

Table 3.3: Computed concentration (μM) of nitrite production in three horses.

	CO	LPS	IL	PMB	PMB+LPS	PMB+IL
HORSE 1	5.35	10.4	26.5	3.46	9.76	12.9
HORSE 2	3.99	11.5	28.2	4.87	11.8	15.8
HORSE 3	4.76	9.54	35.8	5.02	13.7	19.8
MEAN	6.86	27.3	8.84	3.91	5.5	14.3
SD	1.84	11.1	2.03	1.02	3.91	4.13

with control and IL-1 β individually. All treatments (except lipopolysaccharide and polymyxin B) showed a significant difference in nitrite production in comparison with control ($4.7 \pm 0.68 \mu\text{M}$) (Figure 3.5a, Table 3.3). The highest supernatant concentration of nitrite for IL-1 β (alone) treatment (Figure: 3.5a) and all treatments were significantly different in nitrite production in comparison with IL-1 β alone ($30.2 \pm 4.93 \mu\text{M}$) (Figure 3.5a). Lipopolysaccharide (alone) showed similar nitric oxide production as control (Figure 3.5a).

3.3.1.3 PGE₂ analysis

The calibration curve fit for PGE₂ production is shown in Appendix 2 (Figure 4). Figure 3.5b and table 3.4 display data obtained by a competitive ELISA for PGE₂ quantification (n=3). Comparison of PGE₂ production showed there was a significant difference ($P < 0.0001$, F value 19.89, R² value 0.8923, one-way ANOVA) across

the treatments. When compared with control (182 ± 57.6 pg/ml), IL- 1β alone showed significantly higher PGE₂ release

Table 3.4: Computed concentration (pg/ml) of PGE₂ production in three horses.

	CO	LPS	IL	PMB	PMB+LPS	PMB+IL
HORSE 1	118	167	380	104	159	180
HORSE 2	228	122	376	*	122	217
HORSE 3	201	112	314	113	112	203
MEAN	182	134	357	85.4	131	200
SD	57.7	29.1	37.2	40.4	24.6	19

(357 ± 37.2 pg/ml) and also the highest among all treatments (Figure 3.5b). Whereas IL- 1β in the presence of polymyxin B showed significantly lower PGE₂ release (200 ± 20 pg/ml) when compared with IL- 1β (alone) (Figure 3.5b, Table 3.4). PGE₂ production from lipopolysaccharide (in presence and absence of polymyxin B) showed no significant difference with control (134 ± 29.1 and 131 ± 24.6 pg/ml, respectively). All treatments showed a significant difference in PGE₂ release in comparison with IL- 1β (alone) (Figure 3.5b).

3.3.1.4 GAG analysis

3.3.1.4.1 Colorimetry

Calibration curves of GAG production for three horses are shown in Appendix 2 (Figure 5a-c). Comparison of GAG % release showed

there was a significant difference ($P < 0.0001$, F value 19.88, R^2 value 0.8923, one-way ANOVA) across the treatments. Lipopolysaccharide alone (34.7 ± 6.35 % release) and IL-1 β (alone) (46.6 ± 3.55 % release) showed a significant difference in GAG production in comparison with control (18.9 ± 3.17 % release) (Figure 3.5c, Table 3.5). At the same time, all treatments showed a significant difference in GAG breakdown in comparison with IL-1 β (alone) (Figure 3.5c). GAG release from IL-1 β in the presence of polymyxin B was half that of IL-1 β (alone) (Figure 3.5c).

Table 3.5: Computed concertation (%) of GAG release in three horses.

	CO	LPS	IL	PMB	PMB+ LPS	PMB+ IL
HORSE 1	16.5	37.5	45.4	16.4	27.8	20.4
HORSE 2	20.4	39.3	50.7	18.3	30.5	28.5
HORSE 3	22.8	27.5	43.9	13.6	20.5	23.5
MEAN	19.9	34.7	46.6	16.1	26.2	24.1
SD	3.17	6.35	3.55	2.40	5.17	4.08

3.3.1.4.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4 (Figure 3.6 (a-g) according to OARSI. Under 10x magnification, qualitative data of GAG degradation determination is presented here (Figure: 3.6 a-g). Fresh and control cartilage were graded 1 (Figure 3.6 a and b). Polymyxin B, LPS (in the presence and absence of polymyxin B) treated cartilage were graded 2 (Figure 3.6 c, d and

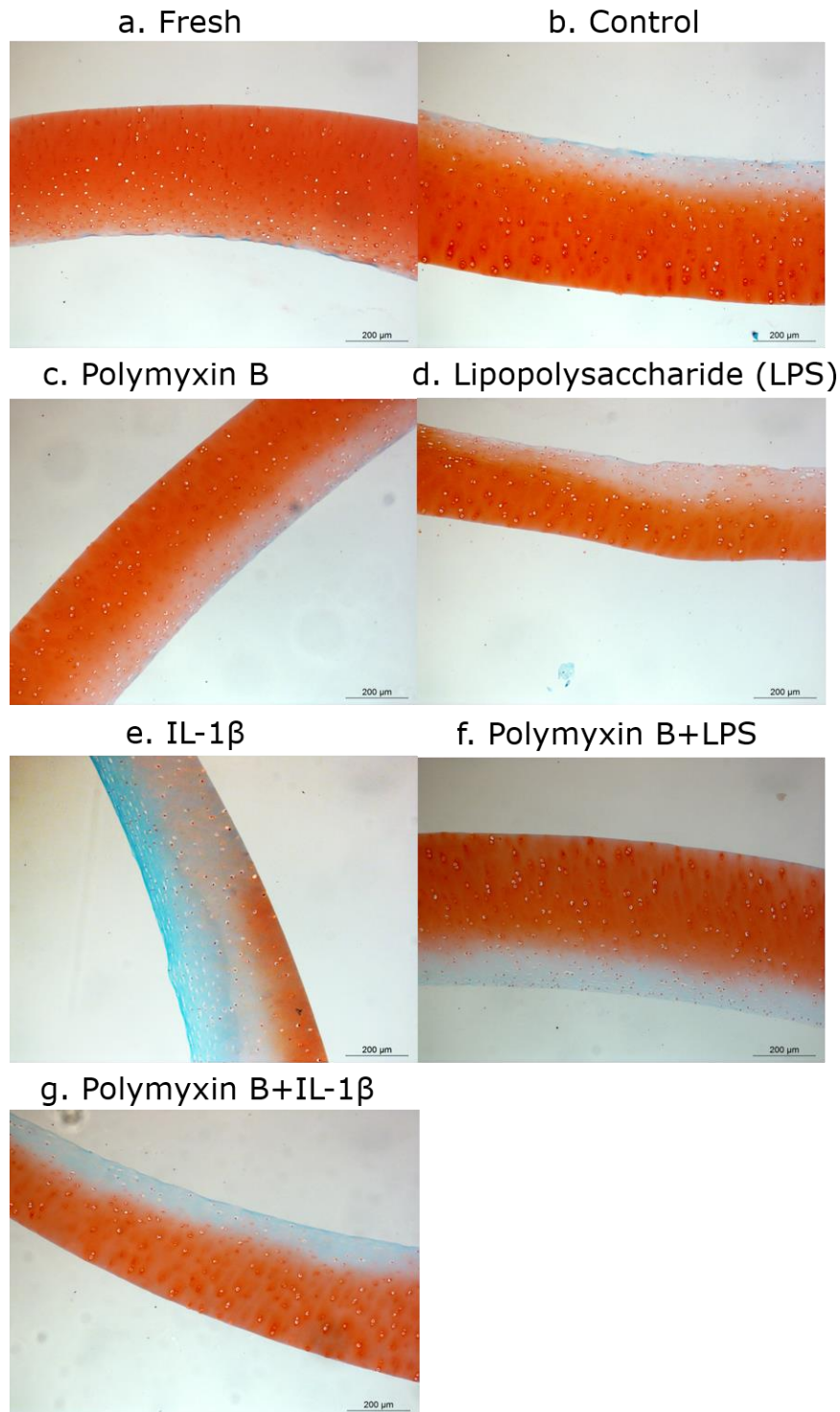


Figure 3.6: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), polymyxin B (PMB) (13 IU/ml) (c), lipopolysaccharide (LPS) (10 ng/ml) (d), IL -1 β (IL) (10 ng/ml) (e), PMB+ LPS (f), PMB + IL 1 β (g). Sections a and b were graded as 1, sections c, d and f were graded as 2, section g was graded as 3 and e was graded as 4 according to OARSI. Magnification x10; scale bars 200 μ m.

f). IL-1 β (alone) treated cartilage was graded as 4 (Figure 3.6 e) and IL- 1 β (in the presence of polymyxin B) treated cartilage was graded 3 (Figure 3.6 g) according to OARSI.

3.4 Estimating LPS activity in equine recombinant IL-1 β product using high dose (10 ng/ml) LPS in the presence of DMSO (1% v/v)

3.4.1 Results

3.4.1.1 Gross morphology from cartilage explant culture

Equine cartilage disks were incubated with culture medium only (Control, CO), vehicle control (control+ 1% DMSO), polymyxin B (PMB) alone (13 IU/ml), lipopolysaccharide (LPS) (10 ng/ml) or IL-1 β (IL) (10 ng/ml) (alone), or LPS or IL in the presence and absence of polymyxin B, all cartilage discs showed no visible gross changes during five days of culture (Figure 3.7).

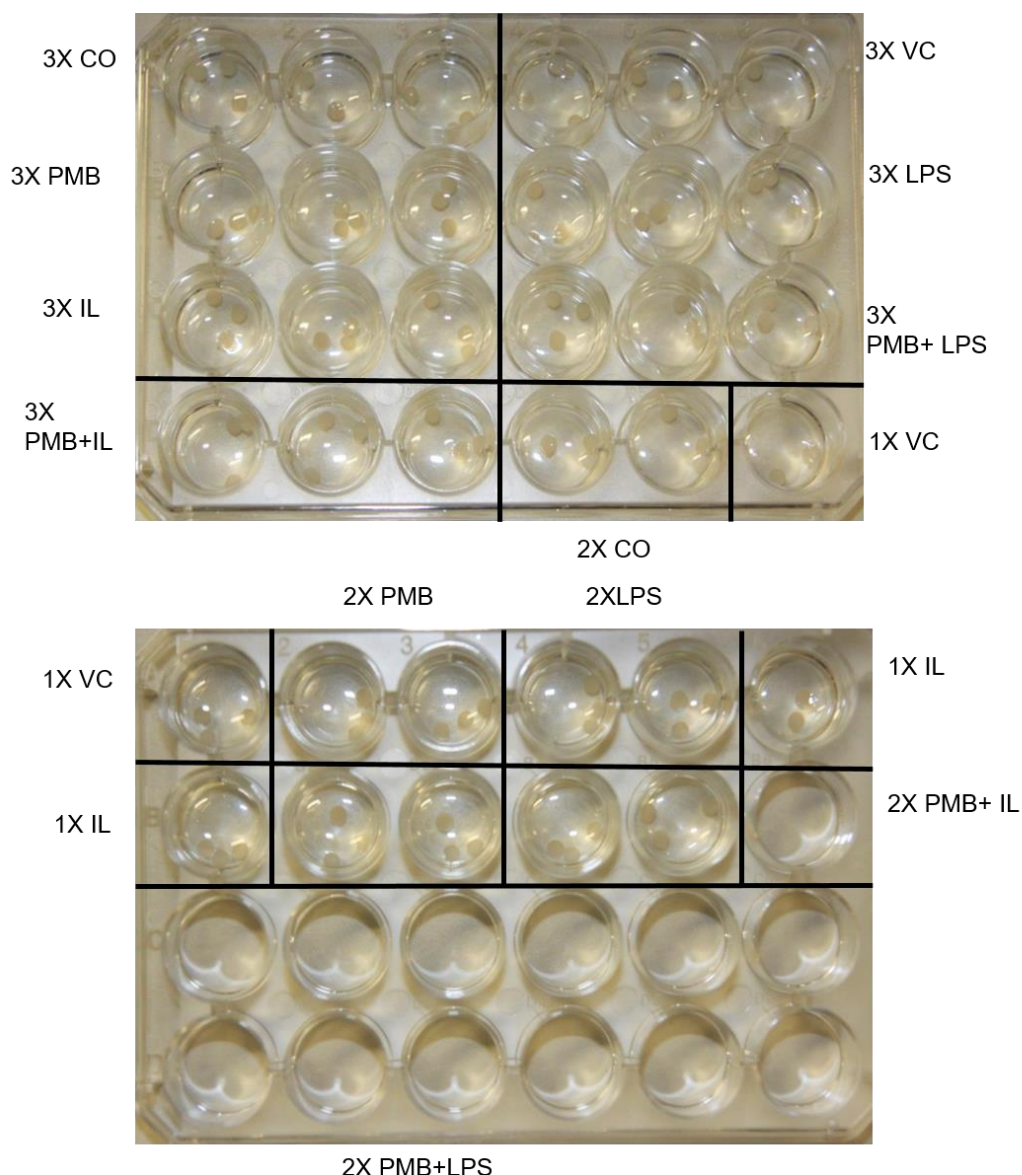


Figure 3.7: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), vehicle control (VC), Polymyxin B (PMB) (13 IU/ml) or Lipopolysaccharide (LPS) (10 ng/ml) and interleukin-1 β (10 ng/ml, IL) in presence and absence of PMB.

3.4.1.1 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 2, Figure 6). Nitrite production for all treatments, in both the presence and the absence of polymyxin B, were compared with

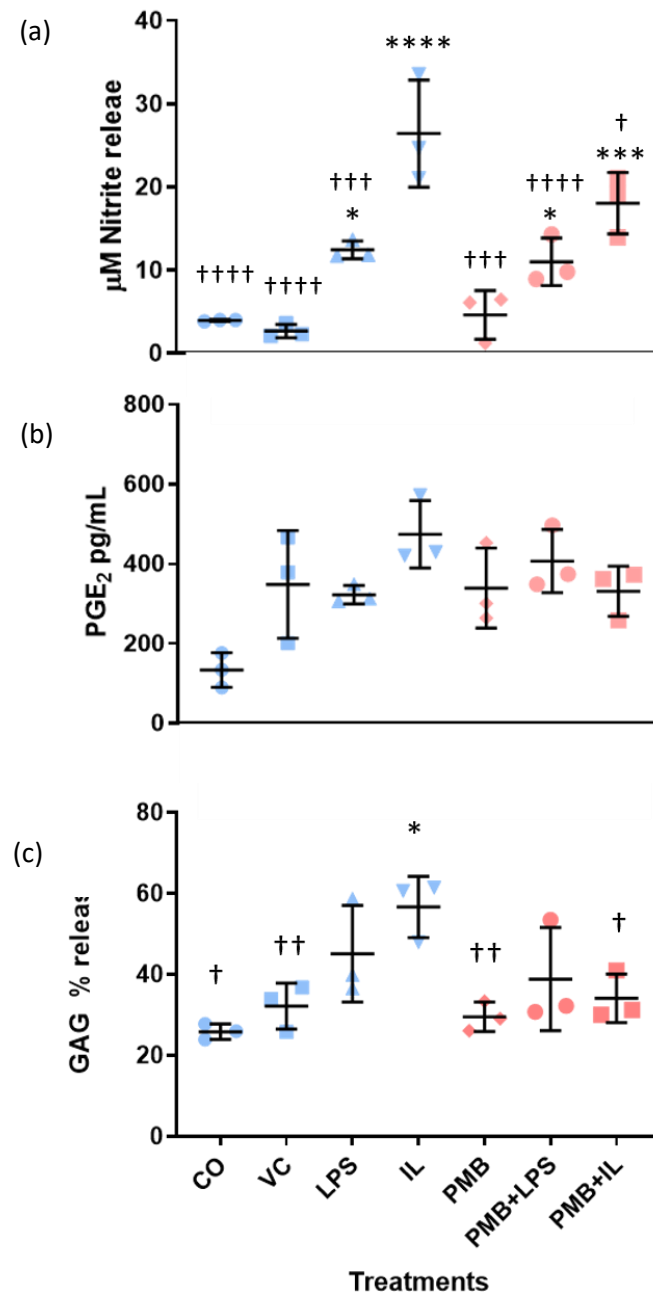


Figure 3.8: Interleukin-1 β (IL) and Lipopolysaccharide (LPS)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were stimulated for 5 days with lipopolysaccharide (LPS) (10 ng/ml), IL -1 β (IL) (10 ng/ml) in presence and absence of polymyxin B (PMB) (13 IU/ml) and PMB alone. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter dot plot with mean and standard deviation (n=3). The release of nitric oxide (nitrite) (n=3) (a), prostaglandin (PGE_2) (n=3) (b), glycosaminoglycan (GAG) (n=3) (c) were measured. One-way ANOVA with Dunnett's post-test was performed. (* $P < 0.05$, *** $P < 0.001$ or **** $P < 0.0001$, significant difference compared to vehicle control; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ or †††† $P < 0.0001$, significantly different to IL alone).

Table 3.6: Computed concentration (μM) of nitrite production in three horses.

	CO	VC	LPS	IL	PMB	PMB+LPS	PMB+IL
HORSE 1	4.03	3.61	11.9	21.1	1.24	8.96	14
HORSE 2	3.85	2.11	13.7	24.7	6.49	14.3	21.1
HORSE 3	4.01	2.33	11.8	33.6	6.12	9.81	19.2
MEAN	3.96	2.68	12.4	26.5	4.62	11	18.1
SD	0.1	0.81	1.07	6.45	2.93	2.87	3.68

vehicle control and IL-1 β individually (n=3). Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 21.3, R^2 value 0.9013, one-way ANOVA) across the treatments. Nitrite production in all treatments was compared with vehicle control and IL-1 β (alone) individually. All treatments (except control and polymyxin B) showed a significant difference in nitrite production in comparison with vehicle control ($2.68 \pm 0.81 \mu\text{M}$) (Figure 3.8a, Table 3.6). The highest supernatant concentration of nitrite was observed for IL-1 β (alone) treated cartilage (Figure: 3.8a) and all treatments were significantly different in nitrite production in comparison with IL-1 β (alone) ($26.5 \pm 6.45 \mu\text{M}$) (Figure 3.8a). Though nitrite production by lipopolysaccharide (alone) was higher than and statistically significant to vehicle control, it was half that of IL-1 β (alone). Nitrite production by polymyxin B ($4.62 \pm 2.93 \mu\text{M}$) was similar to that of vehicle control (Figure 3.8a).

3.4.1.2 PGE₂ analysis

The calibration curve fit for PGE₂ production is shown in Appendix 2 (Figure 7). Figure 3.8b and Table 3.7 display the data obtained by a competitive ELISA for PGE₂ quantification (n=3). None of the treatments showed a significant difference in PGE₂ release in comparison with vehicle control and IL-1 β (alone) (Figure 3.8b, Table 3.7).

Table 3.7: Computed concentration (pg/ml) of PGE₂ production in three horses.

	CO	CV	LPS	IL	PMB	PMB+LPS	PMB+IL
HORSE 1	231	355	143	189	283	100	171
HORSE 2	395	*	122	76	280	122	120
HORSE 3	310	191	178	216	*	*	*
MEAN	312	273	148	161	281	111	145
SD	82.4	116	28.5	74.3	2.29	15.8	36.5

3.4.1.3 GAG analysis

3.4.1.3.1 Colorimetry

Calibration curves of GAG production for three horses are shown in Appendix 2 (Figure 8a-d). Comparison of GAG % release showed there was a significant difference (P value 0.0051, F value 5.224, R² value 0.6913, one-way ANOVA) across the treatments. Only IL-1 β (alone) (56.7 $\pm$ 7.57 % release) showed a significant difference in GAG production in comparison with vehicle control (32.2 $\pm$ 5.6 %

release) (Figure 3.8c, Table 3.8). GAG release was the highest in IL-1 β (alone) treatment (Figure 3.8c) and all treatments (except lipopolysaccharide alone or in presence of polymyxin B, 38.9 $\pm$ 12.7 % release) showed a significant difference in GAG breakdown in comparison with IL-1 β (alone) treatment (Figure 3.8c).

Table 3.8: Computed concertation (%) of GAG release in three horses.

	CO	VC	LPS	IL	PMB	PMB+LPS	PMB+IL
HORSE 1	27.8	33.9	36.6	60.7	29.1	30.8	31.3
HORSE 2	25.9	25.9	58.7	61.4	26.1	53.5	30
HORSE 3	23.9	36.8	39.9	48	33.3	32.3	41
MEAN	25.9	32.2	45.1	56.7	29.5	38.8	34.1
SD	1.93	5.65	11.9	7.57	3.65	12.7	6.02

3.4.1.3.2 Histology

Safranin O and fast green (SOFG) staining characteristics ranged from grade 1 to 4 (Figure 3.9). Under 10x magnification qualitative data of GAG degradation determination is presented here (Figure: 3.9 a-g). Fresh cartilage was graded 1 (Figure 3.9 a). Control, vehicle control, polymyxin B and lipopolysaccharide in presence of polymyxin B treated cartilage were graded 2 (Figure 3.9 b, c d and g). Lipopolysaccharide and IL-1 β treated cartilage were graded as 4 (Figure 3.9 e and f) and IL-1 β in presence of polymyxin treated cartilage was graded 3 (Figure 3.9 h) according to OARSI.

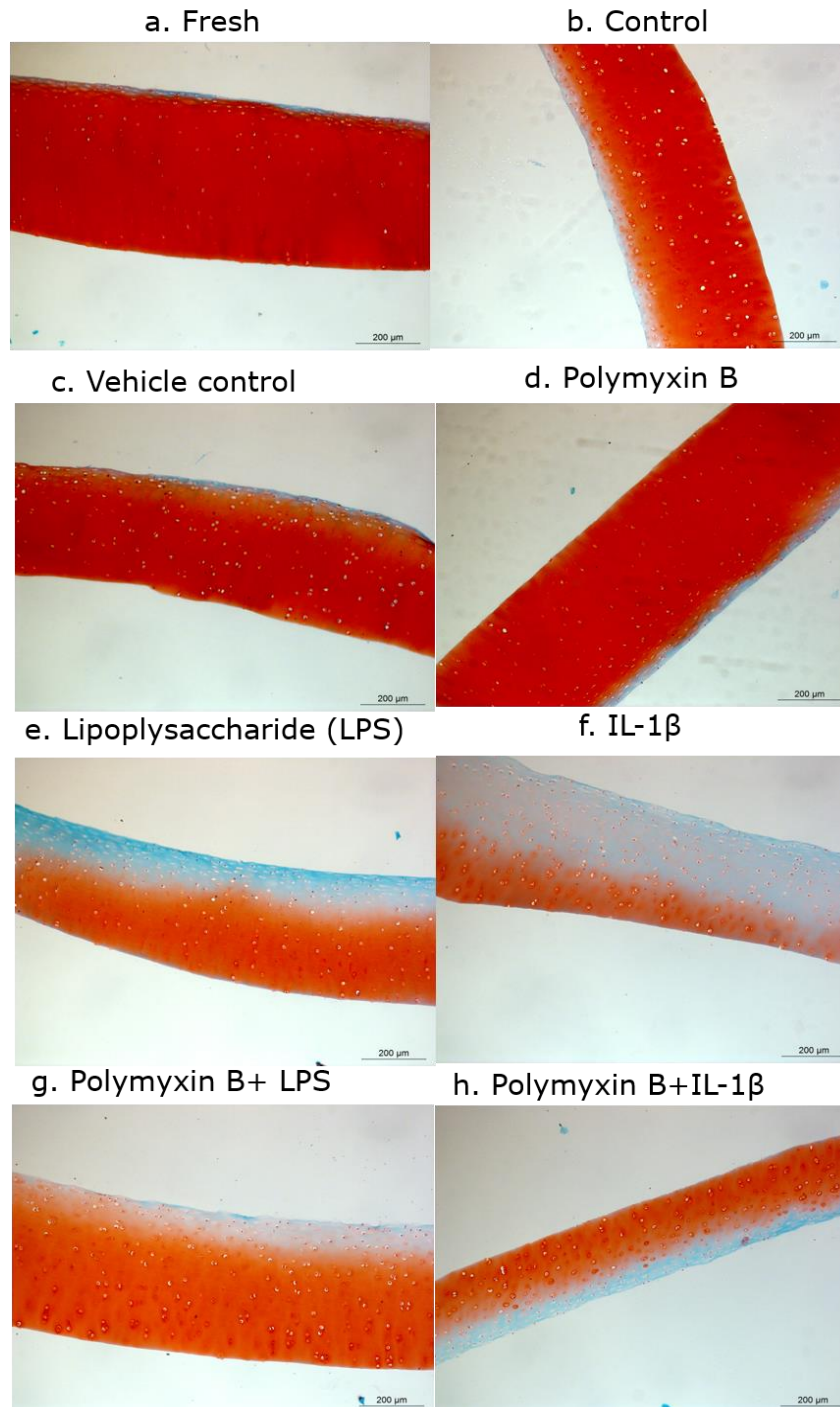


Figure 3.9: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), 1% DMSO in culture media (VC) (c), polymyxin B (PMB) (13 IU/ml) (d), lipopolysaccharide (LPS) (10 ng/ml) (e), IL -1 β (IL) (10ng/ml) (f), PMB+ LPS (g), PMB + IL 1 β (h). Sections a, b, c and d were graded as 1, sections e and g were graded as 2, section f graded as 4 and section h was graded as 3 according to OARSI. Magnification x10; scale bars 200 μ m.

3.5 Discussion

In section 3.2, lipopolysaccharide (100 pg/ml) failed to stimulate inflammatory pathways as monitored by nitric oxide production and GAG breakdown in equine cartilage explant culture. This implies that 1% contamination of lipopolysaccharide in the recombinant equine IL-1 β product does not have any significant consequence effect on the stimulation of the inflammatory pathway. In section 3.3, lipopolysaccharide (10 ng/ml in the absence of DMSO) failed to stimulate nitric oxide and PGE₂ production, however, raised GAG degradation but not as high as IL-1 β (alone). In the presence of DMSO, lipopolysaccharide (10 ng/ml) (Section 3.4) increased nitric oxide production and GAG degradation, but to a lesser extent than IL-1 β (alone). In this chapter, the evidence suggests that the recombinant equine IL-1 β product is not significantly contaminated with the bacterial endotoxin (lipopolysaccharide).

Production of PGE₂ varied for IL-1 β (alone) between the presence and absence of DMSO and in IL-1 β (alone) was not significantly higher than vehicle control in the presence of DMSO. In another literature study, it was observed that DMSO at the concentration range of 0 -1% did not show any effect on COX-1 dependent PGE₂ production in unstimulated (in the absence IL-1 β) Caco-2 cells. Furthermore, these authors (Hollebeeck *et al.*, 2011) also observed

that COX-2 dependent PGE₂ production by IL-1 β was reduced by 1% DMSO in Caco-2 cells. Despite suppressing PGE₂ production by IL-1 β (alone), DMSO at concentrations $\leq 1\%$ was proposed for use in standard screening *in vitro* procedures to study drug transport and did not show any cytotoxicity in Caco-2 cells.(Hollebeeck *et al.*, 2011, Ingels *et al.*, 2004).

In this study, polymyxin B was used as a specific inhibitor for lipopolysaccharide. Surprisingly it was observed that polymyxin B effectively inhibited nitric oxide and GAG production in the presence of IL-1 β . As far as the author is aware, there is no literature evidence showing the inhibitory mechanism of polymyxin B on nitric oxide production and glycosaminoglycan breakdown by IL-1 β .

3.6 Conclusion

The equine recombinant IL-1 β product used in the equine cartilage explant culture had a negligible effect from any LPS. Despite its nature to obscure PGE₂ production, DMSO as a solvent was found appropriate to use for this study. In addition, polymyxin B was observed as an effective inhibitor of inflammasomes, and further study is needed to determine whether it has the potential to treat inflammation as well as osteoarthritis. All further studies throughout the thesis were carried out with IL-1 β (10 ng/ml) as stimulant and

nutraceuticals dissolved in DMSO with a final explant culture DMSO concentration of 1% (v/v).

Chapter 4

Effects of resveratrol and piceatannol in an equine explant cartilage model of inflammation associated with osteoarthritis

4.1 Introduction

Currently, there are no disease modifying osteoarthritis drugs (DMOADs) available. Existing therapies for osteoarthritis (OA) do not target the cause of osteoarthritis but aim to prevent or protect the structural deterioration of a joint. Current therapeutic choices comprise of simple analgesics, such as non-steroidal anti-inflammatory (NSAID) drugs, intra-articular corticosteroid injections and physical therapy (Martin *et al.*, 2007). Phenylbutazone (PBZ) has been the most commonly administered NSAID in horses for the treatment of osteoarthritis. It has a limited safety margin, likely because phenylbutazone inhibits both COX-1 and COX-2 when administered at therapeutic dosages in horses (Doucet *et al.*, 2008). NSAIDs are associated with significant gastrointestinal and cardiovascular toxicity in long term use (Janssens *et al.*, 2008). Corticosteroids are effective in controlling inflammation, but the mechanisms that confer this action are largely unknown (Cato and Wade, 1996). Corticosteroids, such as prednisolone will reduce pain in osteoarthritis at low safe doses only for a short time period (Niemelä *et al.*, 2016). Nitric oxide plays an important role in inflammation as well as osteoarthritis and is produced via inducible nitric oxide synthase (iNOS). S-Ethylisothiurea (EIT) is a potent inhibitor of iNOS and eNOS with little selectivity towards either isoform (Southan *et al.*, 1995), however, S-ethylisothiurea is not

used for treating osteoarthritis. Nowadays nutraceuticals are becoming popular worldwide to treat various diseases including osteoarthritis. Resveratrol (3, 4', 5-trans-trihydroxystilbene) and its analogue piceatannol (3, 3', 4, 5'-trans-trihydroxystilbene) have anti-inflammatory activity (Piotrowska *et al.*, 2012).

In this chapter, it is hypothesized that resveratrol and piceatannol will inhibit IL-1 β induced production of nitric oxide, PGE₂ and also GAG breakdown in an equine cartilage explant culture better than phenylbutazone (NSAID), prednisolone (corticosteroid) and S-ethylisothiourrea (nitric oxide inhibitor).

4.2 Results

4.2.1 Gross morphology after cartilage explant culture

Equine cartilage disks were incubated with prednisolone, phenylbutazone, S-ethylisothiourrea (EIT), resveratrol and piceatannol in an IL-1 β stimulated explant culture for five days. All cartilage discs showed no visible gross changes during five days of culture, except for those with resveratrol and piceatannol treatments. Resveratrol treated cartilage disks (in the presence and absence of IL-1 β) developed a light yellow colour whereas

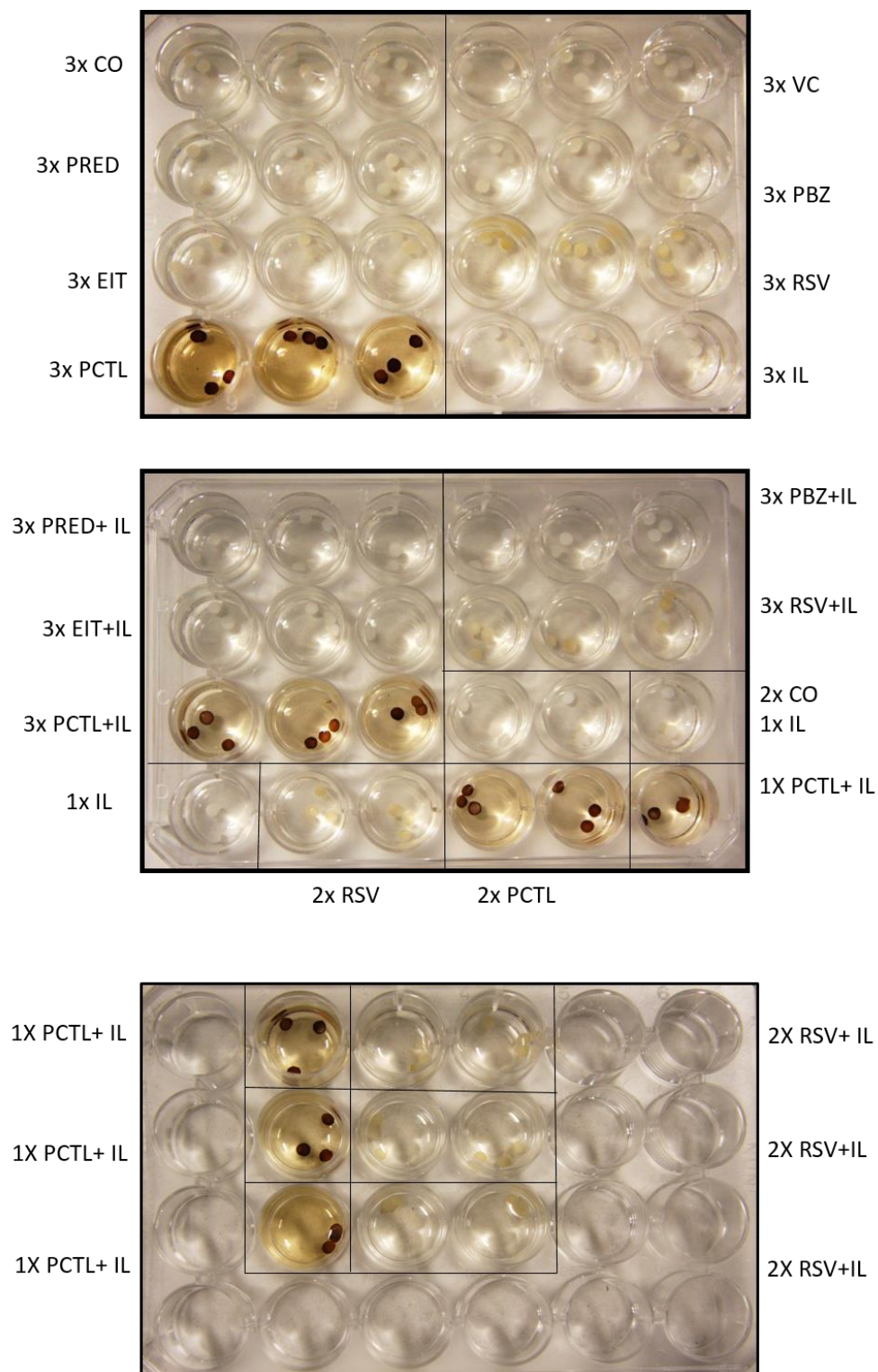


Figure 4.1: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), medium + 1% DMSO (vehicle control, VC) and interleukin-1 β (10 ng/ml, IL) or prednisolone (PRED), phenylbutazone (PBZ), S-ethylisothioureia hydrobromide (EIT), resveratrol (RSV) and piceatannol (PCTL) alone (all at 100 μ g/ml) or in the presence of IL.

piceatannol treated cartilage became a dark coffee colour (in the presence and absence of IL-1 β) (Figure 4.1).

4.2.2 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 3, Figure 1a-g). Nitrite production for all treatments, in both the presence and the absence of IL-1 β , were compared with vehicle control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 12.56, R^2 value 0.5917, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β produced very low nitric oxide levels and showed no significant difference in nitrite production in comparison with vehicle control ($3.76 \pm 1.65 \mu\text{M}$) (Figure 4.2a). All treatments in the absence of IL-1 β produced nitrite levels of approximately one-third of that stimulated by IL-1 β alone ($13.9 \pm 7.22 \mu\text{M}$) and were significantly different in comparison with IL-1 β alone (Figure 4.2a). The highest supernatant concentration of nitrite was observed for IL-1 β (alone) treated cartilage (Figure 4.2a, Table 4.1). Phenylbutazone in the presence of IL-1 β treated supernatant also showed one of the highest nitrite releases ($10.5 \pm 4.98 \mu\text{M}$) which was significantly different from vehicle control (Figure 4.2a). The rest of the treatments in the presence of IL-1 β showed no significant difference with vehicle control-treated supernatant (Figure 4.2a). All treatments in the presence of IL-1 β

Table 4.1: Computed concentration (μM) of nitrite production in nine horses.

	CO	CV	PRED	PBZ	EIT	RSV	PCTL	IL	PRED+IL	PBZ+IL	EIT+IL	RSV+IL	PCTL+IL
HORSE1	5.95	4.51	3.99	4.72	4.10	4.55	4.02	8.53	5.50	8.55	4.09	4.60	4.84
HORSE2	1.93	1.75	1.53	1.99	1.10	1.03	1.18	7.19	4.53	6.83	0.76	2.27	1.58
HORSE3	2.56	0.93	1.15	1.66	0.86	0.93	1.42	8.66	3.77	8.71	1.50	2.00	2.00
HORSE4	2.69	3.09	2.06	2.79	1.70	2.11	2.49	4.19	3.10	4.05	1.91	2.64	3.30
HORSE5	4.59	3.29	2.06	2.69	2.48	2.82	1.91	14.81	5.19	11.63	2.61	6.51	3.57
HORSE6	5.18	4.38	7.39	4.09	3.23	4.77	3.22	19.21	7.80	11.53	3.38	4.21	1.87
HORSE7	5.55	5.54	4.13	3.76	2.95	4.45	3.46	16.22	6.86	8.56	2.43	5.22	3.67
HORSE8	7.90	5.81	3.82	3.51	3.99	3.60	3.89	20.21	8.25	21.76	3.09	8.69	4.51
HORSE9	6.17	4.57	3.56	6.31	3.67	5.96	4.89	26.02	8.75	12.40	3.87	5.35	3.90
Mean	4.72	3.76	3.3	3.50	2.69	3.36	2.94	13.9	5.97	10.5	2.63	4.61	3.25
SD	1.97	1.65	1.91	1.44	1.22	1.75	1.27	7.22	2.03	4.98	1.11	2.17	1.18

(except phenylbutazone in the presence of IL-1 β) produced very low nitrite levels and showed a significant difference in nitrite production in comparison with IL-1 β alone treatment (Figure 4.2a). S-ethylisothiourrea was used as a blocker of nitric oxide inhibition and in the presence of IL-1 β showed the lowest nitrite level ($2.63 \pm 1.11 \mu\text{M}$) among all treatments which was 100% of maximum inhibition (Figure 4.2a). Resveratrol (in the presence of IL-1 β) and piceatannol (in the presence of IL-1 β) treated supernatants showed similar inhibition, 91% and 100% respectively, to S-ethylisothiourrea in the presence of IL-1 β treated supernatant (Figure 4.2a).

4.2.3 PGE₂ analysis

The calibration curve fit for PGE₂ production is shown in Appendix 3 (Figure 2a-i). Figure 4.2b and Table 4.2 display data obtained by a competitive ELISA for PGE₂ quantification (n=9). Comparison of PGE₂ production showed there was a significant difference

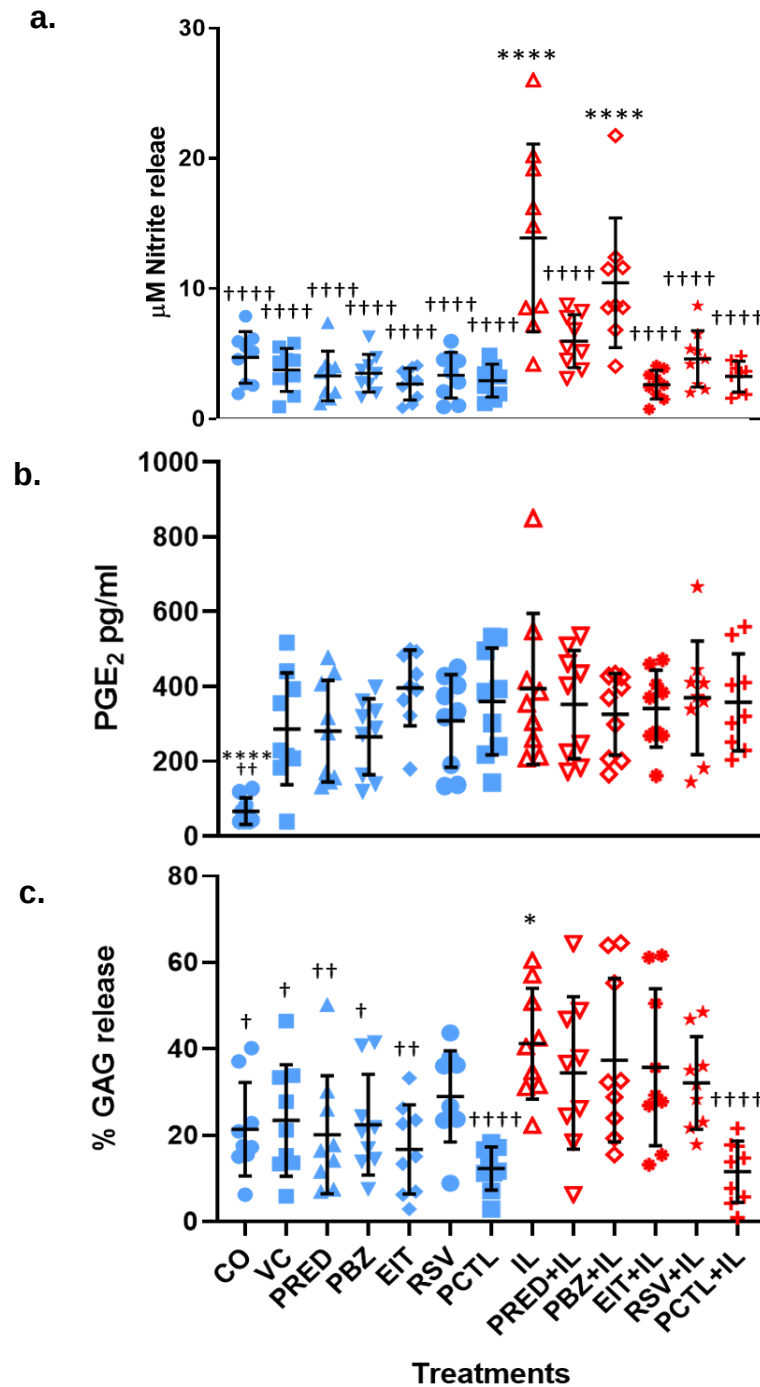


Figure 4.2: Interleukin-1 β (IL)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) alone, or prednisolone (PRED), phenylbutazone (PBZ), ethylisothiouraea hydrobromide (EIT), resveratrol (RSV) and piceatannol (PCTL), all at a concentration of 100 $\mu\text{g}/\text{ml}$, in the presence and absence of IL. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter plots with mean and standard deviation. The release of (a) nitric oxide (nitrite) ($n=9$), (b) prostaglandin (PGE_2) ($n=9$) and (c) glycosaminoglycan (GAG) ($n=9$) were measured and compared using one-way ANOVA with Dunnett's post-test (* $P < 0.05$ or **** $P < 0.0001$, significant difference compared to vehicle control; + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.0001$, significantly different to IL alone).

Table 4.2: Computed concentration (pg/ml) of PGE₂ production in nine horses.

	CO	VC	PRED	PBZ	EIT	RSV	PCTL	IL	PRED+IL	PBZ+IL	EIT+IL	RSV+IL	PCTL+IL
HORSE 1	43.4	229	157	118	493	190	218	260	223	201	161	444	204
HORSE 2	82.9	183	171	162	484	137	240	213	172	166	268	145	229
HORSE 3	43.0	208	146	139	180	134	143	305	183	206	269	181	251
HORSE 4	67.6	356	318	397	400	428	532	417	461	426	406	373	410
HORSE 5	118	393	478	319	498	403	531	386	535	398	459	411	538
HORSE 6	127	517	437	360	433	450	496	849	508	428	471	666	560
HORSE 7	39.0	215	133	333	364	318	302	354	246	370	280	338	302
HORSE 8	39.0	39	408	287	322	334	384	208	434	298	371	360	321
HORSE 9	39.0	440	277	271	391	374	393	548	402	433	384	409	403
MEAN	66.6	287	281	265	396	308	360	393	352	325	341	370	358
SD	35.3	150	136	102	101	124	143	202	145	109	103	152	130

($P < 0.0001$, F value 3.852, R^2 value 0.3077, one-way ANOVA) across the treatments. Only control (53.8 ± 49 pg/ml) showed a very low PGE₂ production which was significantly different in PGE₂ release in comparison with vehicle control (210 ± 188 pg/ml) and IL-1 β (270 ± 272 pg/ml) (Figure 4.2b).

4.2.3.1 GAG analysis

4.2.3.1.1 Colorimetry

Calibration curves of GAG degradation for nine horses are shown in Appendix 3 (Figures 3a-r). Comparison of GAG % release showed there was a significant difference ($P < 0.0001$, F value 5.06, R^2 value 0.3686, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β showed no significant difference in GAG production in comparison with vehicle control (23.4 ± 12.9 % release) (Figure 4.2c). At the same time, all treatments (except

resveratrol) in the absence of IL-1 β showed low GAG breakdown and a significant difference in comparison with IL-1 β (alone) treated cartilage and supernatant (Figure 4.2c). GAG release was the highest in IL-1 β (alone) treated case (41.2 ± 12.8 % release) compared to all other treatments (Figure 4.2c, Table 4.3) and showed a significant difference in GAG release when compared to vehicle control (Figure 4.2c). No treatments in the presence IL-1 β showed a significant difference in GAG breakdown in comparison with vehicle control (Figure 4.2c) though GAG breakdown was higher than vehicle control. Resveratrol treated cartilage (in the presence and absence of IL-1 β) showed lower but non-significant GAG release to IL-1 β alone (Figure 4.2c, Table 4.3). Inhibition of GAG breakdown was 51.3% in the case of resveratrol treated cartilage in the presence of IL-1 β . Only piceatannol in the presence of IL-1 β (11.5 ± 7.12 % release) showed a significant difference in GAG release in comparison with IL-1 β (alone) treated cartilage (Figure 4.2c). Piceatannol treated cartilage (in the presence and absence of IL-1 β) showed the lowest GAG release among all treatments (Figure: 4.2c, Table 4.3) representing almost complete inhibition of GAG breakdown.

Table 4.3: Computed concertation (%) of GAG release in nine horses.

	CO	VC	PRED	PBZ	EIT	RSV	PCTL	IL	PRED+IL	PBZ+IL	EIT+IL	RSV+IL	PCTL+IL
HORSE 1	22.8	13.6	26.0	20.8	26.2	36.1	11.2	34.7	46.8	24.0	27.8	46.8	4.2
HORSE 2	6.2	5.9	7.0	7.5	2.9	36.2	2.9	31.1	18.4	19.3	26.9	35.1	0.9
HORSE 3	15.5	33.4	17.7	16.9	13.4	38.0	16.7	42.5	36.6	32.3	29.3	36.0	7.7
HORSE 4	15.0	33.8	14.2	24.3	15.1	43.7	11.3	40.5	24.4	32.6	35.7	48.5	14.7
HORSE 5	17.5	21.2	16.4	21.7	7.0	23.4	7.1	31.5	26.2	28.8	13.2	17.8	17.7
HORSE 6	20.9	13.4	11.7	14.5	23.5	26.6	11.7	50.8	38.0	55.3	50.5	28.3	5.7
HORSE 7	17.2	15.4	7.5	13.9	6.2	8.9	17.2	22.3	6.2	15.5	15.5	23.1	21.6
HORSE 8	40.2	27.7	30.2	40.7	33.2	23.7	14.1	60.5	48.9	64.5	61.6	21.6	13.9
HORSE 9	37.1	46.5	50.2	41.4	22.6	24.3	18.3	57.1	64.3	63.9	61.1	31.7	17.5
MEAN	21.4	23.4	20.1	22.4	16.7	29	12.3	41.2	34.4	37.4	35.7	32.1	11.5
SD	10.8	12.9	13.7	11.7	10.3	10.6	5	12.8	17.7	18.9	18.2	10.7	7.12

4.2.3.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4. Under 10x magnification, qualitative data of GAG degradation determination is presented in figure 4.3a-g. Fresh cartilage was collected immediately after dissection and then processed and stained with SOFG staining. Control, IL- 1 β , resveratrol (in the presence and absence of IL-1 β) and piceatannol (in the presence and absence of IL-1 β) treated cartilage was collected after 5 days of explant culture. Fresh, control, piceatannol (in the presence and absence of IL-1 β) treated cartilage were graded 1 (Figures 4.3a, b, f and g), IL-1 β (alone) treated cartilage was graded 4 (Figure 4.3c) and resveratrol (in the presence and absence of IL-1 β) (Figures 4.3d and e) treated cartilage was graded 3.

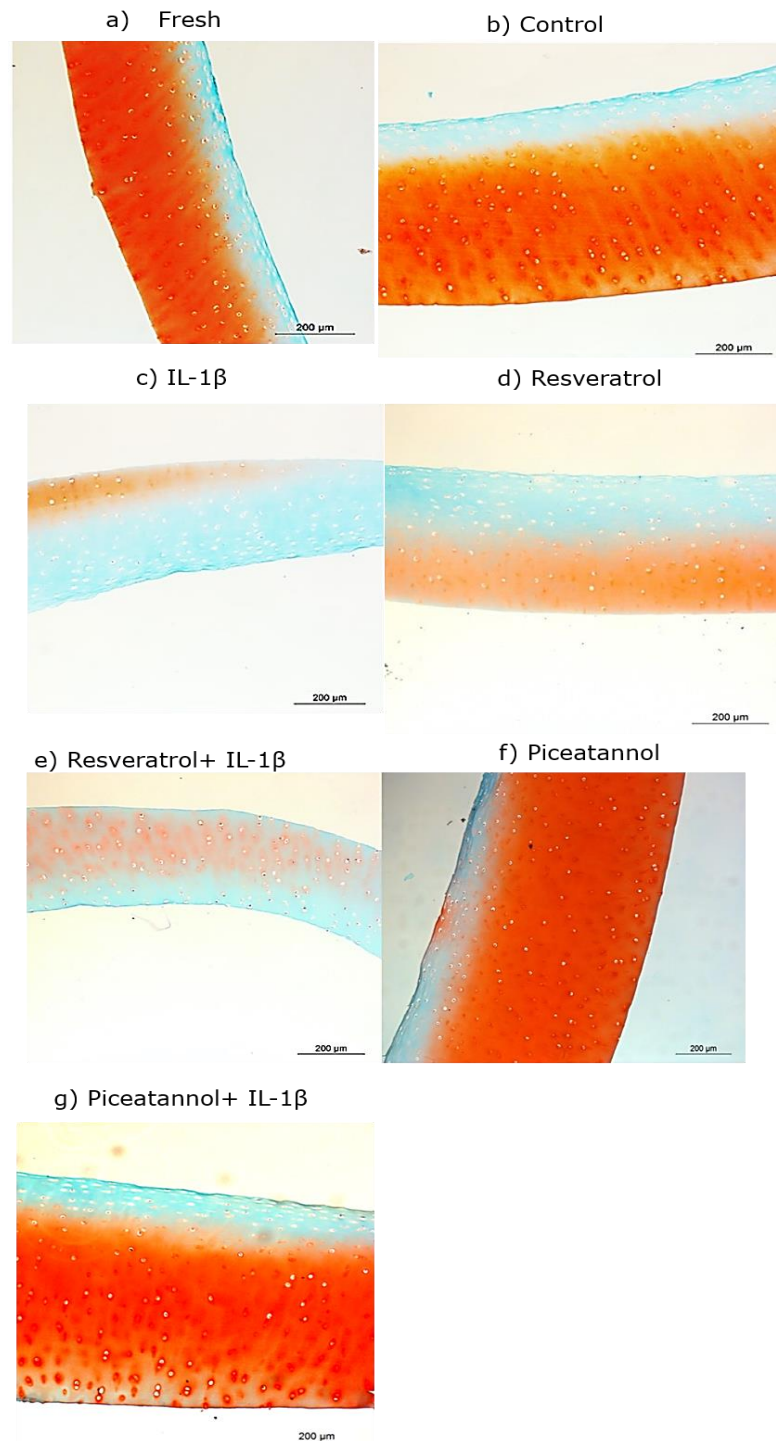


Figure 4.3: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO), (c) interleukin-1 β alone (IL-1 β), (d) resveratrol, (e) resveratrol + IL-1 β , (f) piceatannol and (g) piceatannol + IL-1 β . Resveratrol and piceatannol were used at a concentration of 100 μ g/ml in culture; whereas IL-1 β was used at a concentration of 10 ng/ml. Sections a, b, f and g were graded as 1, section c was graded as 4 and sections d and e were graded as 3 according to OARSI. Magnification x10; scale bars 200 μ m.

4.2.4 Cell viability count

Cell viability count was compared between fresh, control, IL-1 β , resveratrol (in the presence and absence of IL-1 β) and piceatannol (in the presence and absence of IL-1 β) (n=3) (Figure 4.4). Fresh cartilage was collected immediately after dissection and then processed and stained with H and E staining. Control, IL-1 β alone, resveratrol (in the presence and absence of IL-1 β) and piceatannol (in the presence and absence of IL-1 β) treated cartilage was collected after 5 days explant culture followed by H and E staining. Only resveratrol in the presence of IL-1 β (63.1 ± 4.35 % cell viability) showed a significant difference in cell viability in comparison with fresh (83.9 ± 0.98 % cell viability), control (76.8 ± 2.59 % cell viability) and IL-1 β (alone) (75.9 ± 0.69 % cell viability) treated cartilage (Figure 4.4) (Table 4.4).

Table 4.4: Computed cell viability data in three horses.

	FRESH	CO	RSV	PCTL	IL	RSV+IL	PCTL+IL
HORSE 1	84.2	78.3	66.2	69.4	75.1	63.2	74.9
HORSE 2	82.8	73.8	79.2	81.4	76.2	67.4	79.7
HORSE 3	84.6	78.3	79.7	74.5	76.3	58.7	81.9
MEAN	83.9	76.8	75	75.1	75.9	63.1	78.8
SD	0.98	.59	7.69	6.02	0.69	4.35	3.57

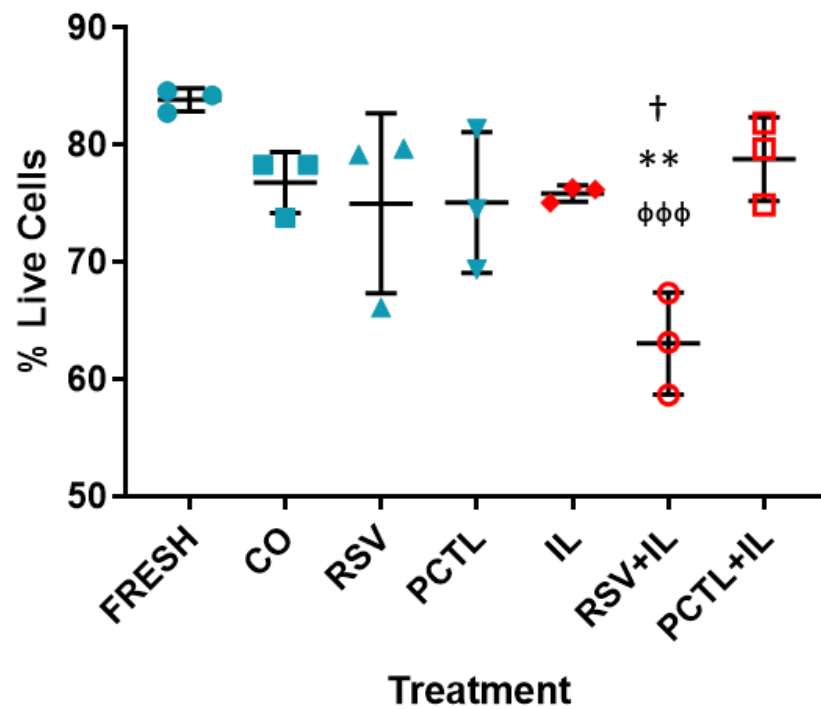


Figure 4.4: Cell viability count of fresh equine cartilage and explant culture. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with resveratrol (RSV), piceatannol (PCTL) at a concentration of 100 $\mu\text{g/ml}$ in presence and absence of IL-1 β (10 ng/ml) (IL) and IL-1 β alone. Control was culture media only (CO). Data are presented as a scatter plot with mean and standard deviation ($n=3$). H & E stained cells were counted in 5 non-overlapping fields of view from 3 sections (the difference between two consecutive sections is 25~50 μm) in each disk. Each treatment comprised of 6 disks; whereas fresh cartilage contained one disk/section. Lowest and highest numbers of cells count in one field of view were 8 and 115 consecutively. One-way ANOVA with Dunnett's test was performed. ($\phi\phi\phi$ $P < 0.001$, significant difference compared to fresh; ** $P < 0.01$, significantly different to control; $\dagger P < 0.05$, significantly different to IL-1 β)

4.3 Discussion

In vitro, nitric oxide exerts detrimental effects on chondrocyte functions, including the inhibition of collagen and proteoglycan synthesis, enhanced apoptosis and inhibition of B1 integrin-dependent adhesion to the extra-cellular matrix (Amin and Abramson, 1998). Reduction (but not significantly) of nitric oxide levels by phenylbutazone was observed in this study. In a previous study, it was observed that nitric oxide reduction by phenylbutazone occurred due to its anti-oxidative properties (Martínez Aranzales *et al.*, 2015). Limited data is available with respect to understanding the precise mechanism(s) of action of various NSAIDs on iNOS synthesis and activity (Tung *et al.*, 2002). In this study, prednisolone significantly inhibited nitric oxide production in the stimulated cartilage explant culture which is similar to previous literature which showed that prednisolone can down-regulate iNOS in mouse macrophages (Chi *et al.*, 2001). A specific inhibitor of iNOS (S-ethylisothiouraea) was used to compare inhibitory effects with that of resveratrol and piceatannol on nitric oxide production. A decrease in nitrite concentration for S-ethylisothiouraea in the presence of IL-1 β treated supernatant also supports the evidence that S-ethylisothiouraea is a potent inhibitor of nitric oxide synthase as shown by Pelletier *et al.* (1998) and Raynauld *et al.* (2003). However, effective inhibition of nitric oxide by resveratrol and

piceatannol was also observed in the present study and may be due to suppression of iNOS expression and subsequent nitric oxide production (Tsai *et al.*, 1999 and Islam *et al.*, 2004). The mode of action of iNOS suppression may be due to the ability of these stilbenoids to inhibit NF- κ B dependent gene expression (Holmes-McNary and Baldwin, 2000). Another possible way inhibition of nitrite by resveratrol may occur is via scavenging nitric oxide which may indicate inhibition via a non-gene expression pathway (Chan *et al.*, 2000).

In this study, a significant difference in PGE₂ production was not observed between vehicle control and IL-1 β alone. An increase in PGE₂ production in the vehicle control compared to the control appears to be due to the 1% DMSO vehicle which supports the observation by Hollebeeck *et al.* (2011) that in unstimulated Caco-2 cells, COX-1-derived PGE₂ production was increased by DMSO. There was no significantly higher PGE₂ production found in the case of IL-1 β treated cartilage compared to vehicle control and one possible explanation may be due to DMSO reducing COX-2 derived PGE₂ production as this has been observed in stimulated Caco-2 cells (Hollebeeck *et al.*, 2011a).

In this chapter, there was no remarkable inhibition of GAG degradation observed by phenylbutazone, prednisolone and S-

ethylisothiourea which is similar to previous literature (Augustine & Oleksyszyn, 1997; De Grauw *et al.*, 2014). There was a clear reduction in GAG degradation for the piceatannol treated cartilage both in the colorimetric analysis and the histological studies. Whilst the mechanism is unclear for the inhibition of GAG degradation, piceatannol has previously been shown to be a non-receptor Tyrosine kinase inhibitor and inhibitor of ADAMTS-4 and ADAMTS-5 (Lauerfelds *et al.*, 2009). Also, piceatannol has been shown to downregulate the transcription factor STAT-3 (Kumari *et al.*, 2005) in a single cell clone of AK-5 (BC-8 cells) and also stimulates osteogenesis through effects on bone morphogenetic protein-2 production in immortalized fetal osteoblasts (hFOB), and osteosarcoma cells (MG-63) (Chang *et al.*, 2006). In contrast, resveratrol treated (in the presence and absence of IL-1 β) cartilage showed one of the highest GAG releases in both the colorimetric and histological studies and suggests that resveratrol failed to inhibit GAG degradation at 100 μ g/ml. This is contrary to the observation that resveratrol was found to be effective in inhibiting GAG degradation in a canine cartilage explant model (Williamson *et al.*, 2006) at a low dose (1 μ g/ml). It has also been observed in another study that resveratrol antagonizes the IL-1 β mediated stimulation of ADAMTS-4 gene expression in monolayer nucleus pulposus (NP) culture (Li *et al.*, 2008). One possible reason that resveratrol (in the presence and absence of IL-1 β) failed to inhibit GAG degradation

was the concentration used in this study was either a too high or too low.

It was proposed by Cudic *et al.* (2009a) that there are some unique binding behaviours of piceatannol and resveratrol. Firstly, they questioned which structural features within the compound frameworks were important for aggrecanase or MMP inhibition. Secondly, they questioned if these substrates can be utilized to differentiate active site binding molecules from exosite binding molecules. Previous studies (Cudic *et al.*, 2009a; Lauer-fields *et al.*, 2009) showed that inhibition of aggrecanases and MMPs by piceatannol requires all four hydroxyl groups to be present and unmodified. It has been demonstrated by Demeule *et al.* (2000) that resveratrol, which possesses one fewer hydroxyl group than piceatannol, is not an inhibitor of MMP-2 or MMP-9. A previous study showed that MMP-2 and -9 activities increase in all aseptic joint disorders (Brama *et al.*, 1998).

It has been shown by Thomas *et al.* (2007) that in equine articular cartilage chondrocytes, apoptosis is positively associated with the degree of cartilage matrix damage, however, the present study conflicts with these results. In section 4.2.5, IL-1 β and resveratrol in the absence of IL-1 β treated cartilage showed very high GAG release but the cell viability count was not significantly different to

control cartilage. This finding also does not support the literature evidence that in bovine articular cartilage an increase in GAG release is associated with decreased cell viability or vice versa (Natoli and Athanasiou, 2008). In this study the high GAG release along with high cell viability count observed for resveratrol in the absence of IL-1 β and IL-1 β (alone) may support the evidence showed by Jeffrey *et al.* (1995) that in bovine articular cartilage chondrocytes are remarkably resilient to tissue disruption. Another possible reason for high cell viability by resveratrol may be due to an improvement of cell survival showed by Li *et al.* (2008) in bovine monolayer nucleus pulposus (NP) culture. Moreover, it was also observed that in human articular chondrocytes resveratrol caused cell proliferation and no toxicity in a wide range of concentrations (Liu *et al.*, 2014). In this study greater cell death by resveratrol in the presence of IL-1 β treated cartilage may indicate a synergistic effect of resveratrol along with IL-1 β on chondrocyte toxicity by an unknown mechanism.

4.4 Conclusion

Piceatannol had a better inhibitory effect on nitric oxide release and GAG degradation in an IL-1 β stimulated cartilage explant model compared to phenylbutazone, prednisolone, S-ethylisothiourea. Resveratrol had a better inhibitory effect than phenylbutazone, prednisolone and S-ethylisothiourea on nitric oxide production but

failed to inhibit GAG degradation. Though the inhibitory effect of resveratrol on nitric oxide production was similar to piceatannol, GAG degradation inhibition was more effective with piceatannol than resveratrol. Overall cell viability count in all treatments was similar other than resveratrol in the presence of IL-1 β treated cartilage. Effects of IL-1 β (alone) and resveratrol in the absence of IL-1 β on GAG degradation and cell death supported the impression that apoptotic cell death and subsequent cell loss may not be related to GAG degradation and therefore is not a major contributor in the early stages of osteoarthritis (Li *et al.*, 2008). This study suggests that GAG degradation is one of the major pathways in the progression of osteoarthritis and that nitric oxide release and cell viability have minor roles in the early stages of osteoarthritis. The mechanism of PGE₂ production appears to be obscured by 1% DMSO. Piceatannol may have the potential to be a disease modifying drug for the treatment of osteoarthritis. The potential role of resveratrol as a disease modifying osteoarthritis drug is still unclear from this study, however, this may be due to the concentration of resveratrol used. In order to have a better understanding of the potency of resveratrol and piceatannol in the cartilage explant model, a dose-response study is required.

Chapter 5

Dose-dependent anti-inflammatory effects of resveratrol in cartilage explant model of equine osteoarthritis

5.1 Introduction

Resveratrol (trans-3, 4-trihydroxystilbene) is a natural phytoalexin (polyphenolic compound) found in the skin of red grapes, peanuts and plums (Alarcón *et al.*, 2005). It shows beneficial health effects, including antioxidant, anti-inflammatory, anti-ageing, cardioprotective and neuroprotective activities (Brisdelli *et al.*, 2009). Resveratrol can suppress the activation of NF- κ B (Manna *et al.*, 2000) and downregulate inflammatory gene products such as COX-2, 5-LOX, IL-1 β , and IL-6 (Aggarwal *et al.*, 2004) which all play a crucial role in arthritis. It has been shown by Liu *et al.* (2010) that in porcine cartilage explants resveratrol suppressed the accumulation of advanced glycation end products (AGEs) leading to induced expression of iNOS, COX-2, production of nitric oxide and PGE₂. It was observed that resveratrol can inhibit interleukin-1 (IL-1)-induced expression of COX-2 and production of PGE₂ through suppressing NF- κ B activity in human chondrocytes (Shakibaei *et al.*, 2008). There is limited clinical evidence available for the effect of resveratrol in osteoarthritis. It has been reported by Elmali *et al.* (2005) that the anti-inflammatory properties of resveratrol could suppress anterior cruciate ligament transaction-induced experimental osteoarthritis in rabbits. In an *in vivo* mice study, resveratrol has been observed to reduce the protein expression of the cartilage-degrading enzymes MMP13 and ADAMTS5 and thus

delayed articular cartilage degradation (Qin *et al.*, 2017). Moreover, it was observed by Collins *et al.* (2015) that in rat articular chondrocyte culture, resveratrol can prevent IL-1 β induced GAG release and cell loss in an acidic condition similar to the low oxygen state of mature chondrocytes. In Chapter 4, it was observed that resveratrol can effectively inhibit nitric oxide production but not GAG degradation at 100 μ g/ml when stimulated with IL-1 β in equine cartilage explant culture. One possible explanation for the lack of inhibition of GAG degradation may be due to the concentration (100 μ g/ml) used.

It is hypothesized that resveratrol can inhibit IL-1 β stimulated (a) nitric oxide in a dose dependent manner and (b) GAG degradation at either a lower or higher dose than 100 μ g/ml in equine cartilage explant culture. The aim of this Chapter is to investigate a range of doses of resveratrol to inhibit IL-1 β stimulated nitric oxide, PGE₂ production and GAG degradation.

5.2 Results

5.2.1 Gross morphology after cartilage explant culture

Cartilage disks were treated with resveratrol 300 μ g/ml (1.31 mM), 100 μ g/ml (0.44 mM), 30 μ g/ml (0.13 mM), 10 μ g/ml (43.8 μ M), 1

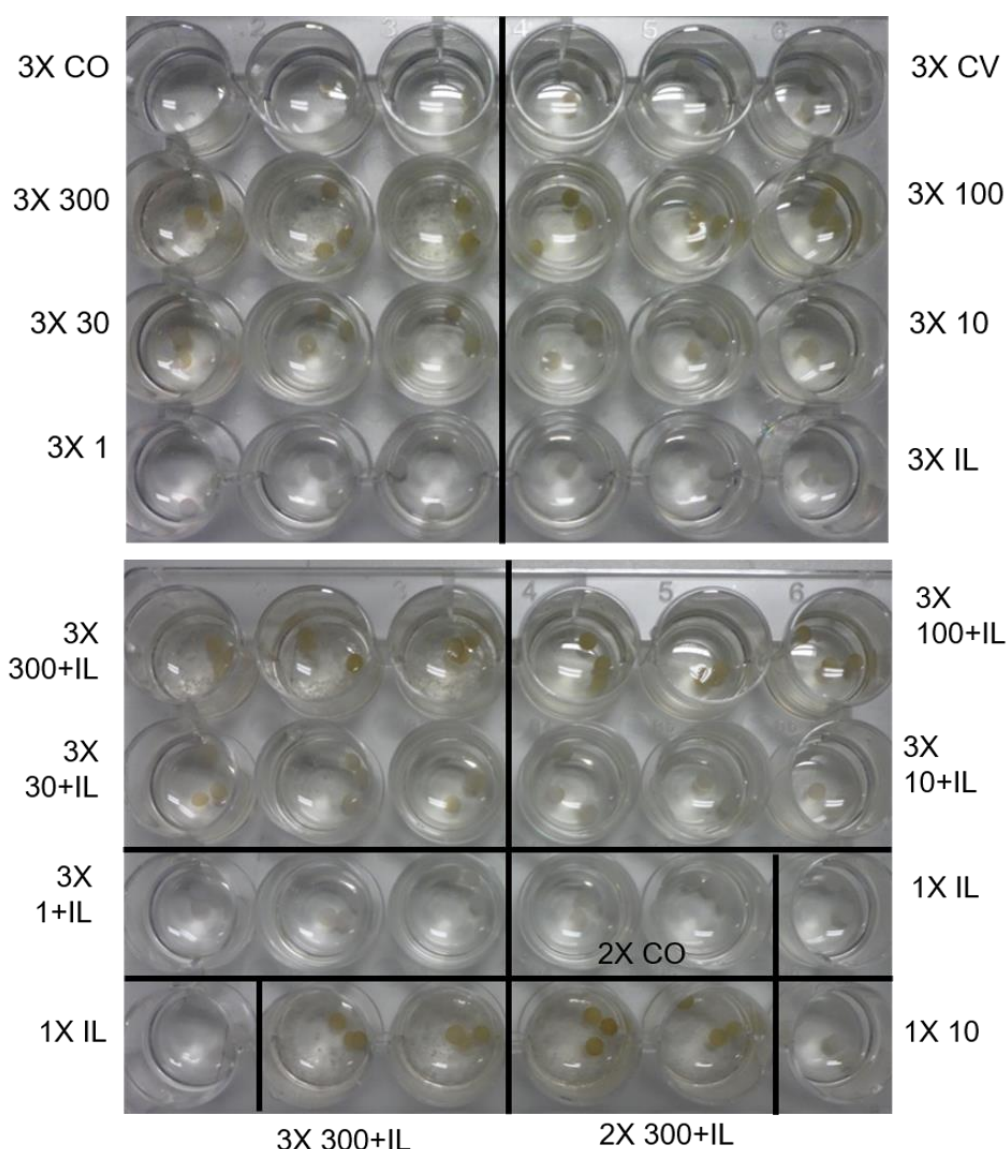


Figure 5.1: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), medium + 1% DMSO (vehicle control, VC) and interleukin-1 β (10 ng/ml, IL) or resveratrol 300 μ g/ml (300), 100 μ g/ml (100), 30 μ g/ml (30), 10 μ g/ml (10), 1 μ g/ml (1) alone or in the presence of IL.

μ g/ml (4.38 μ M) in the presence and absence of IL-1 β , control (medium only), vehicle control (Medium+1% DMSO) and IL-1 β (alone). All cartilage discs showed no visible gross changes during five days of culture, except for those with resveratrol 300 μ g/ml, 100 μ g/ml and 30 μ g/ml treatments. The 300 μ g/ml concentration

in the presence of IL-1 β treated cartilage disks developed the darkest yellow colour among all treatments. Whereas, 100 μ g/ml resveratrol (in the presence and absence of IL-1 β) and 30 μ g/ml resveratrol (in the presence and absence of IL-1 β) showed a lighter colour than 300 μ g/ml resveratrol in presence of IL-1 β consecutively (Figure 5.1).

5.2.2 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 4, figure 1a-b). Nitrite production in all treatments in the presence and absence of IL-1 β were compared with vehicle control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 9.376, R^2 value 0.6338, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β showed low levels of nitrite production and no significant difference in nitrite production in comparison with vehicle control (2.92 ± 2.43 μ M nitrite) (Figure 5.2a, Table 5.1). All treatments in the absence of IL-1 β were significantly different in nitrite production in comparison with IL-1 β alone (Figure 5.2a). The highest supernatant concentration of nitrite was observed for IL-1 β (alone) (8.30 ± 3.38 μ M nitrite) treated cartilage (Figure 5.2a, table 5.1). In the presence of IL-1 β , 1 μ g/ml resveratrol treatment also showed one of the highest nitrite releases (5.37 ± 1.23 μ M nitrite)

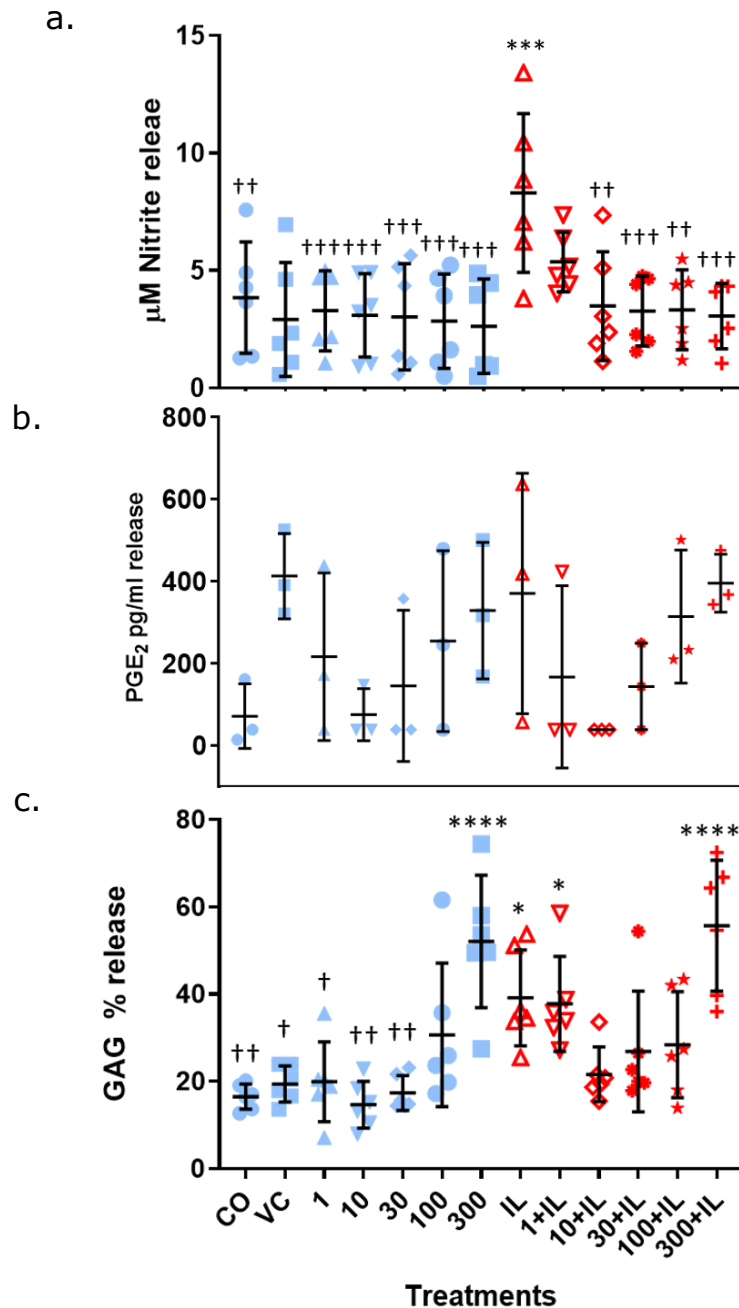


Figure 5.2: Interleukin-1 β (IL)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) alone, or resveratrol 300 $\mu\text{g/ml}$ (300), 100 $\mu\text{g/ml}$ (100), 30 $\mu\text{g/ml}$ (30), 10 $\mu\text{g/ml}$ (10), 1 $\mu\text{g/ml}$ (1), in the presence and absence of IL. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter plots with mean and standard deviation. The release of (a) nitric oxide (nitrite) ($n=6$), (b) prostaglandin (PGE_2) ($n=3$) and (c) glycosaminoglycan (GAG) ($n=6$) were measured and compared using one-way ANOVA with Dunnett's post-test (* $P < 0.05$, *** $P < 0.001$ or **** $P < 0.0001$, significant difference compared to vehicle control; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ or †††† $P < 0.0001$, significantly different to IL alone).

Table 5.1: Computed concentration (μM) of nitrite production in six horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	1.29	0.58	2.09	0.92	0.60	0.51	0.51	10.45	6.38	2.38	1.55	2.53	1.05
HORSE 2	1.36	1.09	1.07	1.03	1.08	1.64	0.94	3.80	5.18	1.91	1.99	1.18	2.01
HORSE 3	3.66	1.89	2.18	3.24	1.37	1.11	0.99	13.4	4.04	1.15	2.28	1.89	2.55
HORSE 4	4.90	4.63	5.00	4.92	5.64	5.24	4.88	6.22	4.43	3.05	4.41	4.38	4.09
HORSE 5	4.29	2.35	4.71	3.52	4.35	3.93	3.97	7.06	4.79	5.10	4.77	5.49	4.33
HORSE 6	7.58	6.96	4.69	4.90	5.15	4.68	4.47	8.86	7.37	7.35	4.66	4.49	4.34
MEAN	3.85	2.92	3.29	3.09	3.03	2.85	2.63	8.30	5.37	3.49	3.28	3.33	3.06
SD	2.37	2.43	1.71	1.78	2.26	2.01	2.01	3.38	1.27	2.32	1.49	1.70	1.39

(Figure 5.2a) which was not significantly different with either vehicle control or IL-1 β (alone) (Figure 5.2a). The rest of the treatments in the presence of IL-1 β showed no significant difference with vehicle control-treated supernatant (Figure 5.2a).

5.2.3 PGE₂ analysis

The calibration curve fit for PGE₂ production is shown in Appendix 4 (figure 2). Figure 5.2b and Table 5.2 display the data obtained by a competitive ELISA for PGE₂ quantification (n=3). Comparison of PGE₂ production showed there was a significant difference (P value 0.0875, F value 1.874, R² value 0.4638, one-way ANOVA) across the treatments. None of the treatments showed a significant difference in PGE₂ release in comparison with vehicle control and IL-1 β (alone) (Figure 5.2b).

Table 5.2: Computed concentration (pg/ml) of PGE₂ production in three horses.

	CO	VC	1	10	30	100	300	IL	1+ IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	14.4	322	172	149	358	480	318	56.9	39	39	250	502	368
HORSE 2	39	391	39	39	39	246	501	419	39	39	39	233	476
HORSE 3	162	527	439	39	39	39	169	637	424	39	144	210	344
MEAN	71.9	413	217	75.6	146	255	329	371	167	39	144	315	396
SD	79.2	104	204	63.4	185	221	167	293	222	0.0	105	162	70.6

5.2.4 GAG analysis

5.2.4.1 Colorimetry

Calibration curves used to determine GAG production for horses 1-6 are shown in Appendix 4 (Figures 3a-l). Comparison of GAG % release showed there was a significant difference ($P < 0.0001$, F value 9.376, R^2 value 0.6338, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β (except resveratrol 100 μ g/ml and 300 μ g/ml) showed low GAG breakdown and no significant difference in GAG production in comparison with vehicle control (19.4 ± 4.15 % GAG release) (Figure 5.2c, Table 5.3). At the same time, all treatments (except resveratrol 100 μ g/ml and 300 μ g/ml) in the absence of IL-1 β showed a significant difference in GAG breakdown in comparison with IL-1 β (alone) treated cartilage ($39.2 \pm 11\%$ GAG release) (Figure 5.2c). Treatments with IL-1 β (alone), 1 μ g/ml resveratrol in the presence of IL-1 β (48.1 ± 18.4 % GAG release) and 300 μ g/ml resveratrol in the presence of IL-1 β

(45.4±13.9% GAG release) showed no inhibition of GAG breakdown with a significant increase in GAG breakdown in comparison with vehicle control (Figure 5.2c). Though cartilage treated with IL-1 β (alone) showed a significantly higher GAG release when compared to vehicle control, the release was lower than 300 μ g/ml resveratrol in the presence of IL-1 β (Figure 5.2c). In the presence of IL-1 β , 10 μ g/ml resveratrol and 30 μ g/ml resveratrol showed the lowest GAG release among all of the treatments which were not significantly different than IL-1 β alone (Figure 5.2c).

Table 5.3: Computed concentration (%) of GAG release in six horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	20.2	17.9	18.9	18.4	14.5	61.7	74.4	53.8	58.6	33.6	26.5	42.0	72.5
HORSE 2	12.7	16.7	17.1	22.8	23.2	35.7	53.6	25.5	38.8	15.5	17.9	43.4	54.7
HORSE 3	16.5	13.6	20.5	7.88	14.4	23.7	49.5	34.5	32.3	20.9	22.7	25.8	64.4
HORSE 4	17.0	24.0	7.16	15.2	15.7	19.9	27.5	33.8	34.0	18.7	19.7	18.0	36.0
HORSE 5	19.1	20.5	35.6	13.1	21.7	17.2	58.0	51.2	27.3	19.5	54.4	13.9	39.7
HORSE 6	13.7	24.0	20.4	10.6	14.8	26.0	49.6	36.2	35.9	21.5	20.0	27.4	66.8
MEAN	16.5	19.4	19.9	14.7	17.4	30.7	52.1	39.2	37.8	21.6	26.9	28.4	55.7
SD	2.91	4.15	9.15	5.39	3.97	16.5	15.2	11.0	10.9	6.25	13.8	12.1	15.0

5.2.4.2 Histology:

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4 (Figure 5.3). Under 10x magnification, qualitative data of GAG degradation determination is presented in Figure 5.3a-i. Fresh, control, 10 μ g/ml resveratrol treated cartilage were graded 1 (Figures 5.3 a, b and d), 1 μ g/ml resveratrol and 10 μ g/ml

resveratrol in the presence of IL-1 β treated cartilage were graded 2. The IL-1 β , 1 μ g/ml resveratrol in the presence of IL-1 β and 300 μ g/ml resveratrol in the presence of IL-1 β treated cartilage (Figures 5.3 e, f and i) and 10 μ g/ml resveratrol in the presence of IL-1 β treated cartilage were graded as 4 (Figure 5.3 g) according to OARSI.

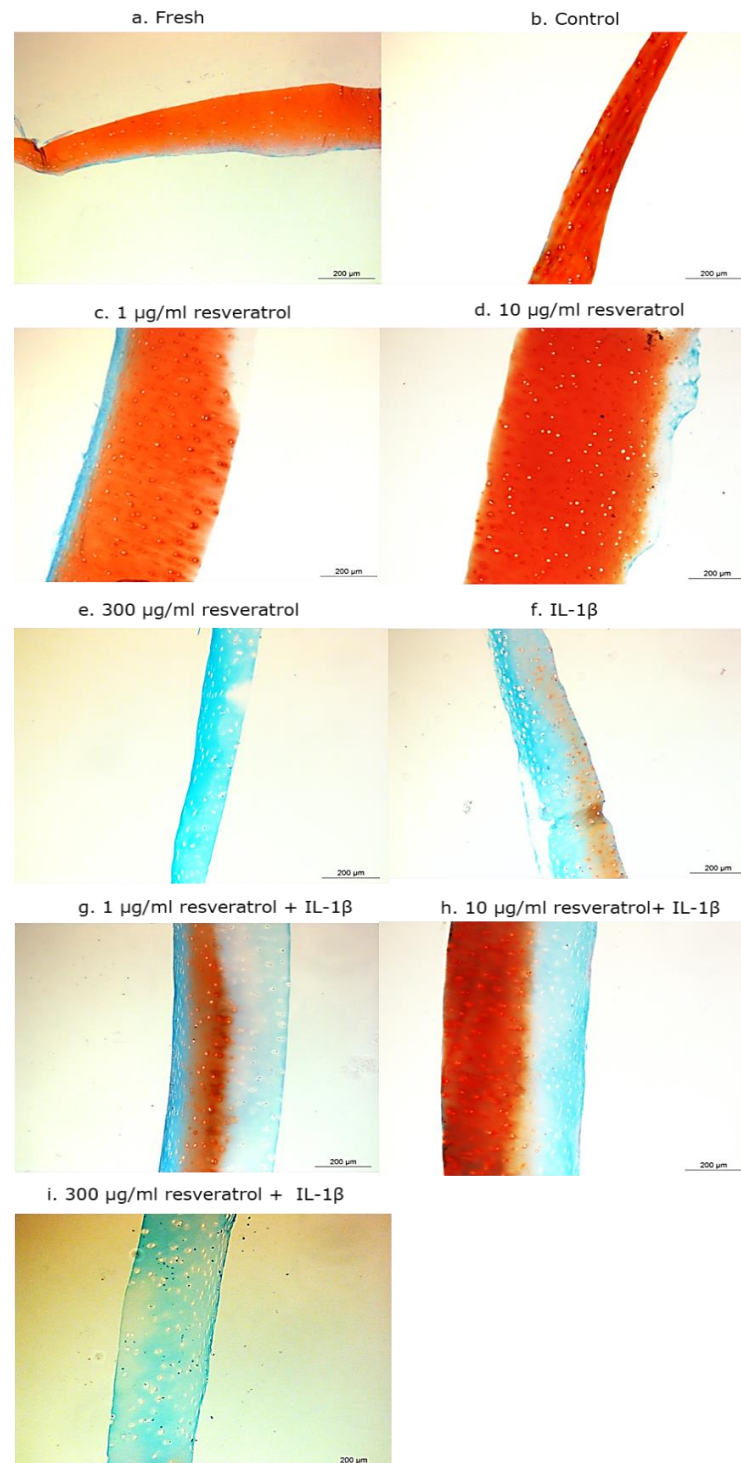


Figure 5.3: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), 1 µg/ml resveratrol (c), 10 µg/ml resveratrol (d), 300 µg/ml resveratrol (e), IL-1 β (f), 1 µg/ml resveratrol + IL-1 β (g), 10 µg/ml resveratrol + IL-1 β (h) and 300 µg/ml resveratrol + IL-1 β (i); IL-1 β was used at a concentration of 10 ng/ml. Sections a, b, and d were graded as 1, section c and h were graded as 2 and sections e, f and i were graded as 4 and section g was graded as 4 according to OARSI. Magnification x10; scale bars 200 µm.

5.3 Discussion

Minimal nitrite production was observed for resveratrol at all concentrations in the absence of IL-1 β . Resveratrol in the presence of IL-1 β at a concentration range of 1-300 μ g/ml inhibited nitric oxide production approximately 54.5%-97%. This indicated that even in low concentrations resveratrol could exert an anti-inflammatory response. This finding also supports previously published evidence that in the presence of IL-1 β , resveratrol at concentrations of 1-5 μ g/ml significantly reduced iNOS expression and nitric oxide production in a dose-dependent manner in rat chondrocyte culture (Lei *et al.*, 2012). This may be an indication that resveratrol is effective to reduce nitric oxide production from both chondrocytes and extracellular matrix.

Similar to previous observations, a significant difference of PGE₂ production was not observed between vehicle control and IL-1 β . Production of high PGE₂ in unstimulated culture and inhibition of PGE₂ production in IL-1 β stimulated explant by 1% DMSO is supported by Hollebeeck *et al.* (2011).

In chapter 4, resveratrol showed one of the highest GAG degradations both in the presence and absence of IL-1 β although the cell viability count with resveratrol alone was similar to the

control and IL-1 β . This raised the question of whether the concentration of resveratrol used in the previous chapter (100 μ g/ml resveratrol) was too low for the inhibition of GAG breakdown. However, it was observed that a much higher concentration of resveratrol (300 μ g/ml resveratrol, in the presence and absence of IL-1 β) was required to produce a higher amount of GAG degradation compared to IL-1 β alone. It has been observed previously that resveratrol (11.4 μ g/ml) in the presence of IL-1 β in human chondrocytes increased the total GAG release compared to both baseline and IL-1 β alone (Vonk *et al.*, 2011). However, the differences between the current study and Vonk *et al.* (2011) may be due to the species and tissue involved. In this study, resveratrol inhibited GAG breakdown with an optimal concentration range of 10-30 μ g/ml in the presence of IL-1 β in equine cartilage explant culture which was also supported by the histology studies.

5.4 Conclusion

In this chapter, resveratrol was found to be an effective nitric oxide inhibitor with an IC₅₀ between 1-10 μ g/ml. The effect of resveratrol on PGE₂ production was ambiguous due to the role of the vehicle used. The pharmacodynamic effect of resveratrol on GAG degradation at different concentrations indicates that resveratrol has a small effective window (10-30 μ g/ml) for inhibiting GAG

degradation in IL-1 β stimulated equine cartilage explant culture. Cell viability was not counted for the dose-dependent effect of resveratrol in this chapter. Cell viability count could be effective in understanding the relationship between cell death and GAG breakdown both in the presence and absence of IL-1 β . From this current dose-response study, it might be said that resveratrol was effective to inhibit all inflammatory effects monitored within equine cartilage explant culture at 10-30 $\mu\text{g/ml}$. To understand effects of resveratrol on inflammatory responses related to osteoarthritis in equine cartilage explant culture, more studies are required to observe effects on cell viability count in a dose-dependent manner, NF- κ B activity and PGE₂ production using a different vehicle.

Chapter 6

Dose-dependent anti-inflammatory effects of piceatannol in cartilage explant model of equine osteoarthritis

6.1 Introduction

Piceatannol (3, 3', 4', 5'-trans-trihydroxystilbene) is a naturally occurring hydroxylated analogue of resveratrol. It has been found in various plants, including grapes, passion fruit, white tea, and Japanese knotweed. Piceatannol has anti-oxidative, anticancer and anti-inflammatory properties (Piotrowska *et al.*, 2012). It has been shown that piceatannol can inhibit the release of nitric oxide (NO), PGE₂, and pro-inflammatory cytokines in a dose-dependent manner in lipopolysaccharide (LPS)-stimulated BV2 microglia during brain injury (Jin *et al.*, 2006). They also showed that piceatannol can diminish the expression of iNOS and COX-2 mRNA and protein levels. Moreover, piceatannol can suppress the transcription of pro-inflammatory cytokine genes through the NF- κ B signalling pathway (Jin *et al.*, 2006). It has been shown in an *in vivo* mouse model that piceatannol (10 mg/kg/ day oral dose) provided protection against degradation of cartilage and the extracellular matrix by increasing collagen II around chondrocytes, promoting nuclear translocation of Nrf2 and decreasing MMP13 (Tang *et al.*, 2017). As far as the author is aware there is little evidence published on the effects of piceatannol on osteoarthritis.

In chapter 4, piceatannol effectively reduced GAG degradation and nitric oxide in an equine cartilage explant model for osteoarthritis.

There was no detrimental effect of piceatannol on cell viability (100 µg/ml) in the presence and absence of IL-1 β . In this chapter, it is hypothesized that piceatannol can inhibit IL-1 β stimulated nitric oxide and GAG degradation in equine cartilage explant culture in a dose-dependent manner. The aim of this chapter is to investigate a range of doses of piceatannol to inhibit IL-1 β stimulated nitric oxide and PGE₂ production and GAG degradation.

6.2 Results

6.2.1 Gross morphology after cartilage explant culture

Cartilage disks were treated with piceatannol 300 µg/ml (1.23 mM), 100 µg/ml (0.41 mM), 30 µg/ml (0.12 mM), 10 µg/ml (40.9 µM) and 1 µg/ml (4.09 µM) in the presence and absence of IL-1 β (10 ng/ml), control (medium only), vehicle control (Medium+1% DMSO) and IL-1 β (IL-1 β alone). All cartilage discs showed no visible gross changes during five days of culture, except for those with piceatannol 300 µg/ml, 100 µg/ml and 30 µg/ml treatments. Piceatannol 300 µg/ml (in the presence and absence of IL-1 β) treated cartilage disks developed the darkest coffee colour among all treatments. Whereas, piceatannol 100 µg/ml (in the presence and absence of IL-1 β) and

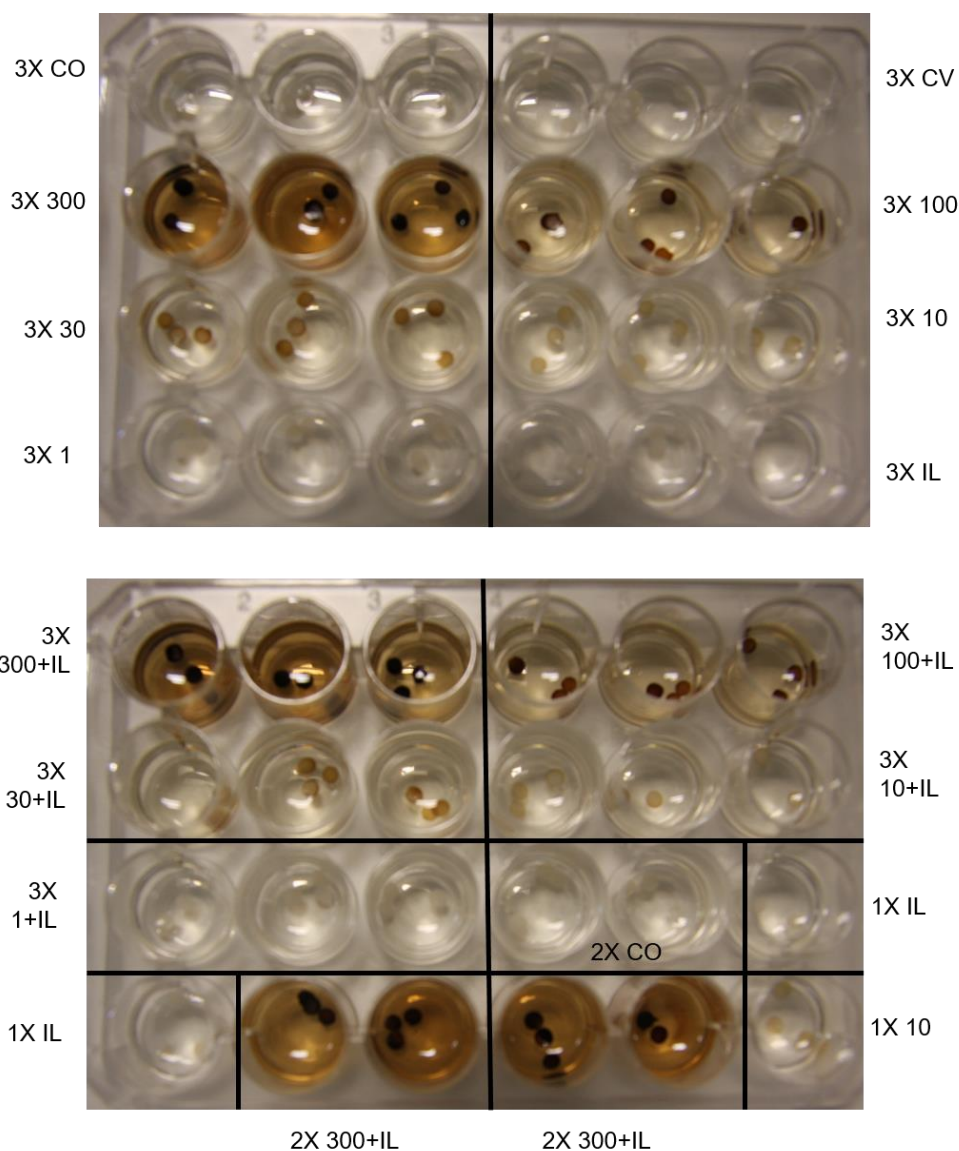


Figure 6.1: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), medium + 1% DMSO (vehicle control, VC) and interleukin-1 β (10 ng/ml, IL) or piceatannol 300 μ g/ml (300), 100 μ g/ml (100), 30 μ g/ml (30), 10 μ g/ml (10), 1 μ g/ml (1) alone or in the presence of IL.

30 μ g/ml (in the presence and absence of IL-1 β) were lighter in colour than 300 μ g/ml piceatannol (in the presence and absence of IL-1 β) consecutively (Figure 6.1).

6.2.2 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 5, Figure 1a-d). Nitrite production for all treatments (in the presence and absence of IL-1 β) were compared with vehicle control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 16.73, R^2 value 0.7554, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β showed no significant difference in nitrite production in comparison with vehicle control ($3.9 \pm 0.43 \mu\text{M}$) (Figure 6.2a, Table 6.1). All treatments in the absence of IL-1 β produced very low nitrite and those nitrite levels were significantly different in comparison with IL-1 β treatment alone (Figure 6.2a). The highest level of nitrite was released in response to IL-1 β treatment ($10.8 \pm 4.76 \mu\text{M}$) (Figure 6.2a). Piceatannol at 1 $\mu\text{g/ml}$ (in the presence and absence of IL-1 β) treatment showed higher nitrite releases ($6.92 \pm 4.53 \mu\text{M}$) (except IL-1 β alone) (Figure 6.2a) among all treatments which are significantly different with vehicle control and IL-1 β (alone) (Figure 6.2a). The rest of the treatments in the presence of IL-1 β showed very low nitrite production and no significant difference with vehicle control-treated supernatant (Figure 6.2a). All treatments in the presence of IL-1 β showed a significant difference in nitrite production in comparison with IL-1 β (alone) treated supernatant (Figure 6.2a). All treatments in

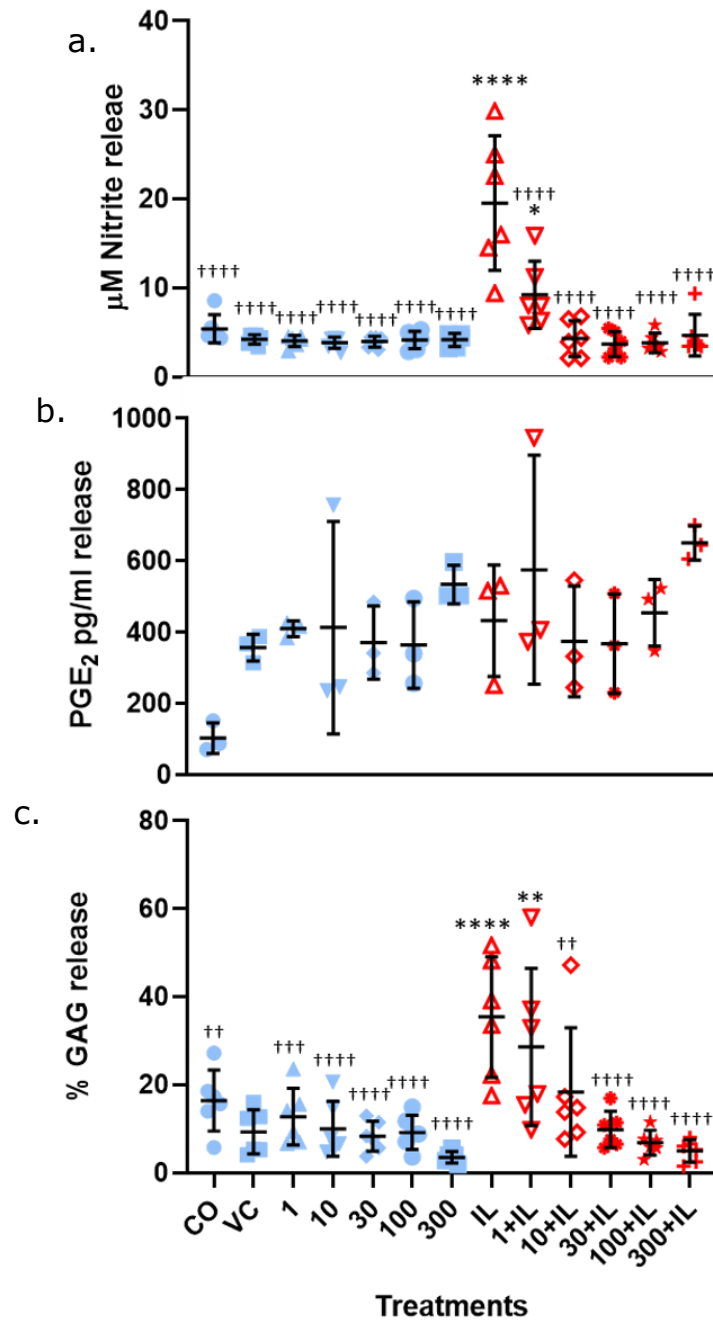


Figure 6.2: Interleukin-1 β (IL)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) alone, or piceatannol 300 $\mu\text{g}/\text{ml}$ (300), 100 $\mu\text{g}/\text{ml}$ (100), 30 $\mu\text{g}/\text{ml}$ (30), 10 $\mu\text{g}/\text{ml}$ (10), 1 $\mu\text{g}/\text{ml}$ (1), in the presence and absence of IL. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter plots with mean and standard deviation. The release of (a) nitric oxide (nitrite) ($n=6$), (b) prostaglandin (PGE_2) ($n=3$) and (c) glycosaminoglycan (GAG) ($n=6$) were measured and compared using one-way ANOVA with Dunnett's post-test (* $P < 0.05$, ** $P < 0.01$ or **** $P < 0.0001$, significant difference compared to vehicle control; ++ $P < 0.01$, +++ $P < 0.001$ or ++++ $P < 0.0001$, significantly different to IL alone).

Table 6.1: Computed concentration (μM) of nitrite production in six horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	5.54	4.40	4.24	4.35	4.18	4.21	4.43	9.42	6.33	4.38	4.02	4.37	4.57
HORSE 2	8.58	4.22	3.66	3.45	3.08	2.88	3.20	16.0	15.9	6.81	5.48	3.32	3.60
HORSE 3	4.37	3.39	2.95	2.77	3.33	3.12	3.28	14.5	5.85	3.95	3.15	3.35	3.66
HORSE 4	4.67	4.73	4.56	4.26	4.44	5.32	4.86	22.6	8.05	2.13	2.21	2.84	3.46
HORSE 4	4.66	3.79	4.36	3.99	4.36	5.01	4.44	29.9	11.3	6.53	5.11	5.84	9.40
HORSE 6	4.67	4.73	4.56	4.26	4.44	4.37	4.86	25.0	8.05	2.13	2.21	3.23	3.46
MEAN	5.42	4.21	4.05	3.85	3.97	4.15	4.18	19.56	9.24	4.32	3.70	3.82	4.69
SD	1.60	0.53	0.63	0.62	0.61	0.98	0.75	7.56	3.77	2.04	1.42	1.11	2.34

Table 6.2: Values from IC_{50} dose-response curves (nitrite)

	Log(IC_{50})	Standard Error of the Log(IC_{50})	IC_{50} ($\mu\text{g}/\text{mL}$)
HORSE 1	-0.06022	0.3144* to 0.01634	0.8705
HORSE 2	0.8216	0.1349	6.631
HORSE 3	-0.6829	0.2801	0.2075
HORSE 4	-0.1079*	2240*	0.78*
HORSE 5	-1.561	1.609	0.02751
HORSE 6	-0.1215*	1859*	0.756*
AVERAGE	-0.2151*	0.09474	0.6095

*Extrapolated value

presence and absence of $\text{IL-1}\beta$ showed a similar amount of nitrite release which was one-third of that of $\text{IL-1}\beta$ (alone). The IC_{50} for nitrite inhibition by piceatannol is approximately 1 $\mu\text{g}/\text{ml}$ (0.6095 $\mu\text{g}/\text{ml}$) (Appendix 5, figure 2g and table 6.2).

6.2.3 PGE₂ detection

The calibration curve fit for PGE₂ production is shown in Appendix 5 (Figure 3). Figure 6.2b and Table 6.3 display the data obtained by a competitive ELISA for PGE₂ quantification (n=9). Comparison of PGE₂ production showed there was a significant difference (P value 0.0439, F value 2.212, R² value 0.5052, one-way ANOVA) across the treatments. None of the treatments showed a significant difference in PGE₂ release in comparison with vehicle control and IL-1 β (alone) (Figure 6.2b).

Table 6.3: Computed concentration (pg/ml) of PGE₂ production in three horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	87.6	368	385	236	286	495	598	252	373	333	363	493	702
HORSE 2	71.2	315	427	247	342	257	503	516	946	245	231	348	645
HORSE 3	152	388	417	757	485	341	503	530	407	546	509	523	606
MEAN	104	357	410	414	371	364	535	433	576	375	368	454	651
SD	42.9	37.8	21.9	298	103	121	54.65	157	321	155	139	93.5	48.1

6.2.4 GAG analysis

6.2.4.1 Colorimetry

Calibration curves of GAG degradation for nine horses are shown in Appendix 5 (Figures 4a-l). Comparison of GAG % release showed there was a significant difference (P<0.0001, F value 7.259, R² value 0.5727, one-way ANOVA) across the treatments. All treatments in

the absence of IL-1 β showed no significant difference in GAG degradation in comparison with vehicle control (9.33 ± 5.01 % release) (Figure 6.2c, table 6.4). At the same time, all treatments in the absence of IL-1 β showed very low GAG degradation and had a significant difference in GAG breakdown in comparison with IL-1 β (alone) treated cartilage (Figure 6.2c). GAG release was the highest in IL-1 β (alone) (35.4 ± 13.7 % release) treated cartilage compared

Table 6.4: Computed concentration (%) of GAG release in six horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	14.0	12.5	14.2	14.7	11.5	7.08	4.04	33.6	15.5	17.3	10.9	5.82	2.47
HORSE 2	27.2	16	23.6	20.6	8.34	11.8	5.55	48.3	58.1	47.3	17	11.6	5.20
HORSE 3	5.75	5.39	7.03	6.55	3.76	3.62	2.73	22.2	9.87	7.70	5.70	3.06	1.51
HORSE 4	15.7	4.13	9.34	4.72	5.70	8.82	1.90	51.7	33	14.8	6.49	7.63	6.02
HORSE 4	17.4	5.25	15.6	7.84	7.73	14.9	4.01	39.2	37.2	13.8	7.64	7.38	6.58
HORSE 6	18.5	12.7	6.73	5.76	12.9	8.80	2.83	17.6	18	9.20	11.4	5.61	8.07
MEAN	16.4	9.3	12.8	10	8.33	9.18	3.51	35.4	28.6	18.4	9.86	6.85	4.97
SD	6.96	5.01	6.47	6.29	3.45	3.89	1.29	13.7	17.9	14.6	4.19	2.84	2.51

Table 6.5: Values from IC₅₀ dose response curves (GAG)

	Log(IC ₅₀)	Standard Error of the Log(IC ₅₀)	IC ₅₀ (μ g/mL)
HORSE 1	-1.087*	0.475	0.08
HORSE 2	1.289	0.332	19.44
HORSE 3	-1.304*	1.224	0.05
HORSE 4	0.01499	0.1629	1.035
HORSE 5	0.2325	0.2218	1.708
HORSE 6	1.029*	2056*	10.7*
AVERAGE	0.3119	0.1937	2.051

*Extrapolated value

to all other treatments and this was significantly different from vehicle control (Figure 6.2c). No treatments in the presence IL-1 β (except 1 μ g/ml piceatannol in the presence of IL-1 β , 28.6 $\pm$ 17.9 % release) showed a significant difference in GAG breakdown in comparison with vehicle control (Figure 6.2c). At the same time, all treatments (except 1 μ g/ml piceatannol in the presence of IL-1 β) showed low GAG degradation and significant difference in GAG production in comparison with IL-1 β (alone) (Figure 6.2c). The IC₅₀ for GAG breakdown inhibition by piceatannol is approximately 2 μ g/ml (Appendix 5, figure 5g and table 6.5).

6.2.4.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4 (Figure 6.3). Under 10x magnification, qualitative data of GAG degradation determination is presented here (Figure 6.3 a-i). Fresh, control, 300 μ g/ml piceatannol (in the presence and absence of IL-1 β) treated cartilage were graded 1 (Figure 6.3 a, b, c and g), 10 μ g/ml piceatannol (in the presence and absence of IL-1 β) and 1 μ g/ml piceatannol (in the absence of IL-1 β) treated cartilage were graded 2 (Figure 6.3 d, e, and h). IL-1 β (alone) treated cartilage was graded 4 (Figure 6.3 h) and 1 μ g/ml piceatannol (in the

presence of IL- β) treated cartilage was graded as 3 (Figure 6.3 i) according to OARSI.

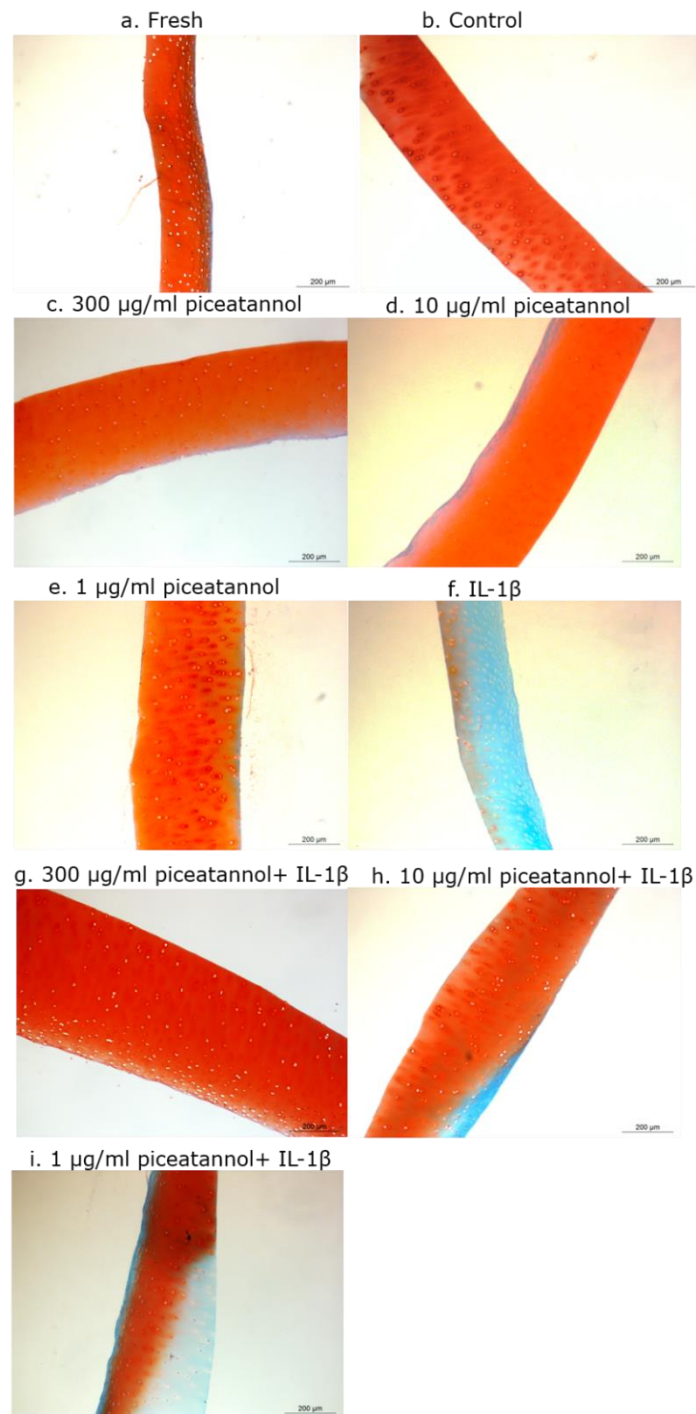


Figure 6.3: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), 300 µg/ml piceatannol (c), 10 µg/ml piceatannol (d), 1 µg/ml piceatannol (e), IL 1 β (f), 300 µg/ml piceatannol + IL 1 β (g), 10 µg/ml piceatannol + IL 1 β (h) and 1 µg/ml piceatannol + IL 1 β (i); IL 1 β was used at a concentration of 10 ng/ml. Sections a, b, g and h were graded as 1, section c, d and e were graded as 2 and sections f were graded as 4 and section i was graded as 3 according to OARSI. Magnification x10; scale bars 200 µm.

6.3 Discussion

In Chapter 4, piceatannol showed better inhibition of all inflammatory responses (nitric oxide production, GAG degradation, and cell viability) in explant equine cartilage culture when compared to phenylbutazone, prednisolone, and S-ethylisothiourea.

In this study, it was observed that piceatannol (from 10 $\mu\text{g/ml}$ and above) inhibited nitric oxide production in the presence of IL-1 β with an IC_{50} of 0.6 $\mu\text{g/ml}$. This finding supports a number of previous studies showing that piceatannol can prevent nitric oxide production in a dose-dependent manner. Piceatannol inhibited iNOS induction and NF- κB activation in LPS-induced mouse macrophage culture which was not due to its cytotoxic action on cells (Islam *et al.*, 2004). Furthermore, piceatannol inhibited nitric oxide production in an LPS-induced mouse macrophage culture by inhibiting iNOS induction and activation of NF- κB but not iNOS enzyme activity (Matsuda *et al.*, 2000; Tadashi *et al.*, 2001).

In this study production of PGE₂ was obscured by 1% DMSO again which is supported by Hollebeeck *et al.*, (2011). Though the effect of piceatannol was masked by DMSO in this study, piceatannol was observed to inhibit PGE₂ production in LPS-induced human mononuclear cells in a dose-dependent manner (1.22-4.88 $\mu\text{g/ml}$)

in the presence of DMSO (concentration not mentioned) (Richard *et al.*, 2005).

In chapter 4, piceatannol (in the presence and absence of IL-1 β) showed the lowest GAG degradation among all treatments. In this Chapter, it was observed that the higher the concentration of piceatannol (in the presence of IL-1 β) the higher the GAG degradation inhibition with an IC₅₀ of 2.1 μ g/ml. A possible mechanism of inhibition of GAG degradation by piceatannol is by the inhibition of aggrecanases, as piceatannol at 1.22 μ g/ml concentration hampers the proteolytic activity of both ADAMTS-4 and ADAMTS-5 in mouse chondrogenic ATDC5 cells (Moncada-pazos *et al.*, 2011). Piceatannol was observed to inhibit ADAMTS-5 in a dose-dependent manner (0-12.2 μ g/ml) particularly at 1.22 μ g/ml and 2.44 μ g/ml concentrations in IL-1 β -stimulated human articular cartilage tissue (P<0.01) (Tang *et al.* 2017). In this study, piceatannol appears to inhibit GAG degradation in the absence of IL-1 β (but not significantly different than vehicle control) and may suggest that piceatannol enhances basal GAG maintenance.

In human articular chondrocyte culture, piceatannol reduced cell viability significantly at ≥ 2.44 μ g/ml at 24 and 48 hours (Tang *et al.*, 2017). However, in Chapter 4, cell viability count after piceatannol treatment of equine explants at a much higher dose

(100 µg/ml) (in the presence and absence of IL-1 β) showed similar cell viability to control after five days of explant culture which contradicts the Tang *et al.* (2017) study. This may be due to species differences or differences between isolated cells and explant cartilage.

6.4 Conclusion

Piceatannol showed significant inhibition of both nitric oxide and GAG degradation at 10-300 µg/ml concentrations in the presence of IL-1 β . Even in the absence of IL-1 β , piceatannol appeared to show GAG degradation inhibition in a dose-dependent manner which may be an indication of basal GAG maintenance. In this study, piceatannol showed a wide effective window (10-300 µg/ml) to inhibit different inflammatory responses monitored in the equine cartilage explant culture. Moreover, with various anti-inflammatory properties, piceatannol showed no cytotoxicity (Chapter 4) at 100 µg/ml concentration. Further investigations are required to observe inhibitory effects of piceatannol on ADAMTS4, ADAMTS5, MMP13, PGE₂ (in a different vehicle) and *in vivo* studies as a DMOAD.

Chapter 7

**Effects of quercetin an
equine cartilage explant model
of inflammation associated with
osteoarthritis**

7.1 Introduction

Quercetin is a flavonoid present in tea, wine, onions, lettuce, cabbage, broccoli, beans, apples, peaches and buckwheat (Williamson and Manach, 2005). Quercetin has been shown to inhibit cytokine and inducible nitric oxide synthase expression through inhibition of the NF- κ B pathway without modification of c-Jun N-terminal kinase activity (both *in vitro* and *in vivo*) in bone marrow-derived macrophages (Comalada *et al.*, 2005). It also inhibited cartilage damage and IL-1 β production in a mouse paw (Haleagrahara *et al.*, 2017). There is limited information concerning the role of quercetin in cartilage and bone remodelling. It has been observed that quercetin qualitatively reduced GAG and inhibited MMP-3 activity in IL-1 β induced bovine chondrocytes (Jackson *et al.*, 2006).

The aim of this study was to compare quercetin with commonly used drugs; phenylbutazone (NSAID), prednisolone (corticosteroid) and the nitric oxide inhibitor marker (S-ethylisothiouraea) in an IL-1 β stimulated equine cartilage explant culture. It was hypothesized that quercetin would (a) inhibit IL-1 β induced production of nitric oxide, PGE₂ and also GAG breakdown in equine cartilage explant culture better than phenylbutazone (NSAID), prednisolone (corticosteroid) and S-ethylisothiouraea (nitric oxide inhibitor marker) and (b) inhibit

IL-1 β stimulated nitric oxide, PGE₂ production and GAG degradation in a dose dependent manner.

7.2 Comparative study on the effect of quercetin in an equine cartilage explant model of inflammation associated with osteoarthritis

7.2.1 Result

7.2.1.1 Gross morphology from cartilage explant culture

Equine cartilage disks were incubated with prednisolone (277 μ M) phenylbutazone (324 μ M), S-ethylisothiouraea (960 μ M), quercetin (409 and 41 μ M) in an IL-1 β stimulated explant culture for five days. The concentration used for all treatments was 100 μ g/ml (except quercetin low, 10 μ g/ml) and IL-1 β was 10 ng/ml. All cartilage discs showed no visible gross changes during five days of culture, except for those with quercetin high dose (QH) (100 μ g/ml) in the presence and absence of IL-1 β treatments which developed a dark yellow colour (Figure 7.1).

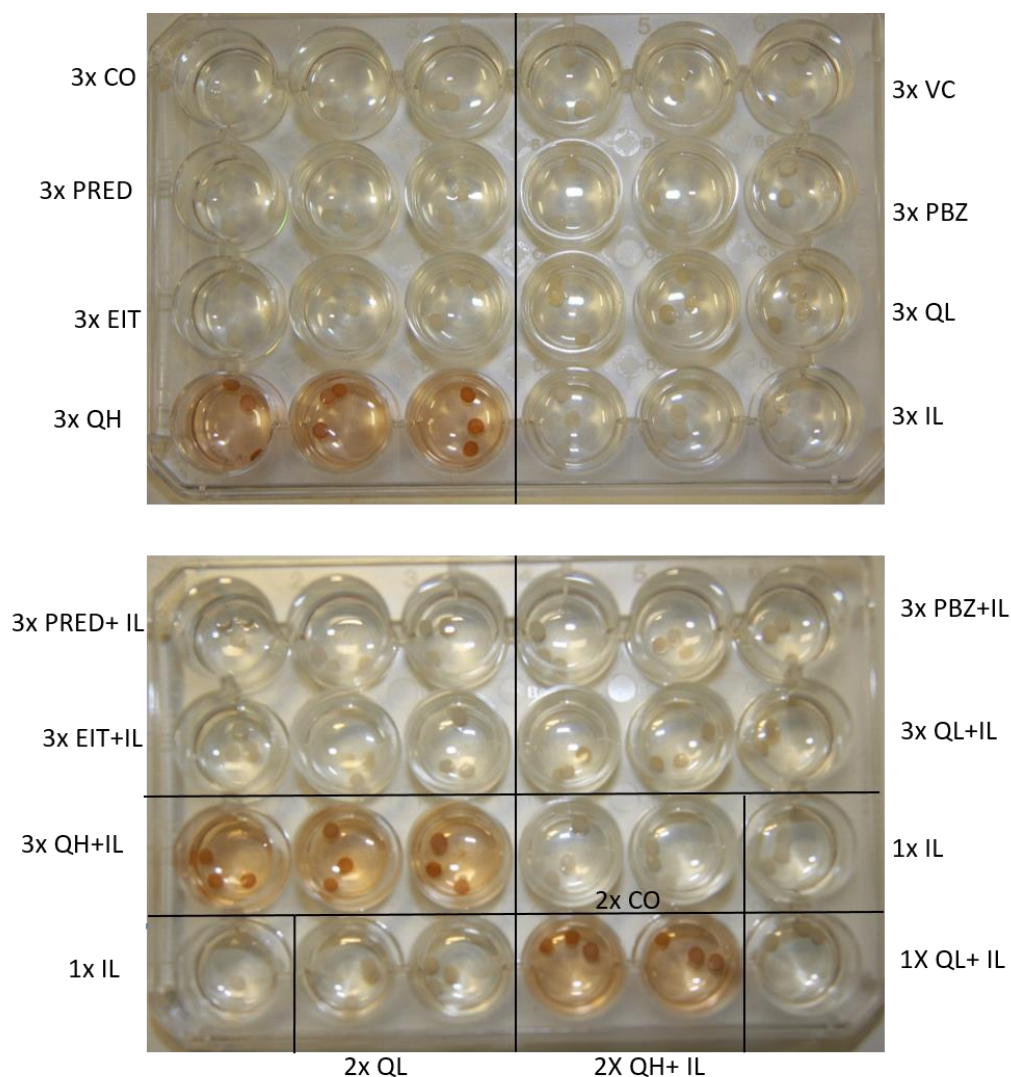


Figure 7.1: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), medium + 1% DMSO (vehicle control, VC) and interleukin-1 β (10 ng/ml, IL) or prednisolone (PRED), phenylbutazone (PBZ), S-ethylisothiurea hydrobromide (EIT), quercetin low dose (QL) and quercetin high dose (QH) alone (all at 100 μ g/ml; except QL at 10 μ g/ml) or in the presence of IL.

7.2.1.1 Nitric oxide analysis

Calibration curves were developed for nitrite production (Appendix 6, Figure 3). Nitrite production for all treatments, in both the presence and the absence of IL-1 β , were compared with vehicle

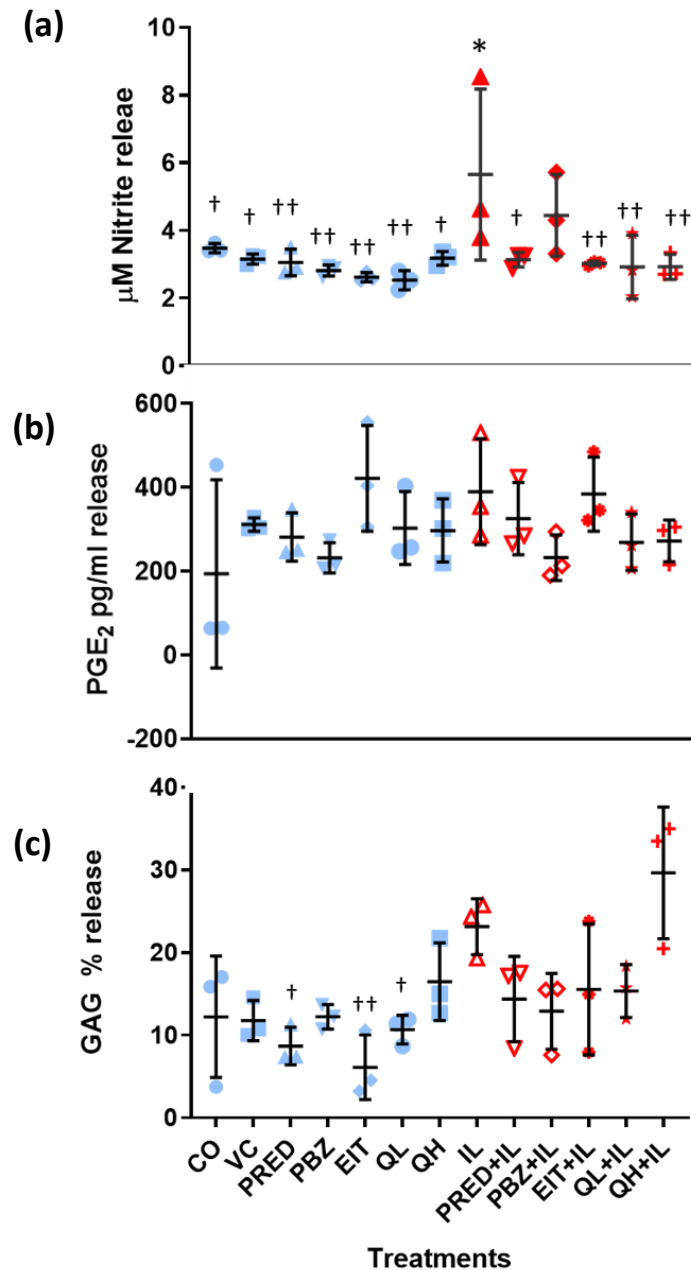


Figure 7.2: Interleukin-1 β (IL)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) alone, or prednisolone (PRED), phenylbutazone (PBZ), ethylisothiourea hydrobromide (EIT), Quercetin high (QH) and quercetin low (QL), all at a concentration of 100 $\mu\text{g}/\text{ml}$ (except QL 10 $\mu\text{g}/\text{ml}$), in the presence and absence of IL. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter plots with mean and standard deviation. Data are presented as scatter plot with mean and standard deviation, mean, minimum and maximum ($n=3$). The release of nitric oxide (nitrite) ($n=3$) (a), prostaglandin (PGE_2) ($n=3$) (b), glycosaminoglycan (GAG) ($n=3$) (c) were measured. One-way ANOVA with Dunnett's post-test was performed. (* $P < 0.05$ or ** $P < 0.01$, significant difference compared to vehicle control; † $P < 0.05$ or †† $P < 0.01$, significantly different to IL alone).

Table 7.1: Computed concentration (μM) of nitrite production in three horses.

	CO	CV	PRED	PBZ	EIT	QL	QH	IL	PRED+IL	PBZ+IL	EIT+IL	QL+IL	QH+IL
HORSE 1	3.41	2.99	3.50	2.88	2.78	2.81	3.21	4.63	3.26	4.30	3.07	2.04	2.70
HORSE 2	3.39	3.22	2.77	2.63	2.51	2.24	2.96	3.79	2.89	3.32	2.95	2.82	2.72
HORSE 3	3.64	3.26	2.89	2.94	2.57	2.53	3.37	8.53	3.26	5.72	3.05	3.91	3.35
MEAN	3.48	3.16	3.05	2.82	2.62	3.18	2.53	5.65	3.14	4.45	3.02	2.93	2.92
SD	0.14	0.15	0.39	0.17	0.14	0.20	0.28	2.53	0.21	1.21	0.07	0.37	0.94

control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference (P value 0.0089, F value 3.02, R^2 value 0.5822, one-way ANOVA) across the treatments. All treatments in the absence of IL-1 β showed very low nitric oxide production and no significant difference with vehicle control ($3.16 \pm 0.15 \mu\text{M}$) (Figure 7.2a, Table 7.1). All treatments in the presence of IL-1 β (except Phenylbutazone in the presence of IL-1 β) showed a significant difference in nitrite production in comparison with IL-1 β (alone) treatment (Figure 7.2a). S-ethylisothiurea was used as a marker for nitric oxide inhibition and in the presence of IL-1 β ($3.02 \pm 0.07 \mu\text{M}$) it showed the lowest nitrite level among all treatments (Figure 7.2a). Quercetin low and high dose (in the presence of IL-1 β) treated supernatants showed similar inhibition to S-ethylisothiurea (in the presence of IL-1 β) (Figure 7.2a).

7.2.1.2 PGE₂ analysis

Calibration curve fit for PGE₂ production is shown in Appendix 6 (Figure 2). Figure 7.2b and table 7.2 display the data obtained by a competitive ELISA for PGE₂ quantification (n=3). Comparison of PGE₂ production showed there was a significant difference (P value 0.2392, F value 1.375, R² value 0.3882, one-way ANOVA) across the treatments. None of the treatments showed a significant difference in PGE₂ release in comparison with vehicle control and IL-1 β (alone).

Table 7.2: Computed concentration (pg/ml) of PGE₂ production in three horses.

	CO	VC	PRED	PBZ	EIT	QL	QH	IL	PRED+IL	PBZ+IL	EIT+IL	QL+IL	QH+IL
HORSE 1	64.1	302	251	216	405	249	220	286	267	214	346	206	216
HORSE 2	454	330	349	274	556	404	371	355	425	295	486	341	298
HORSE 3	65.5	305	246	207	306	258	303	531	286	191	322	262	306
MEAN	195	312	282	232	422	304	298	391	326	233	385	270	273
SD	225	15.7	57.9	36.4	126	87.5	75.5	126	86.2	54.6	88.2	67.7	50.0

7.2.1.3 GAG analysis

7.2.1.3.1 Colorimetry

Calibration curves of GAG production for three horses are shown in Appendix 6 (Figure 3 a-f). Comparison of GAG % release showed there was a significant difference (P value 0.2392, F value 1.375, R² value 0.3882, one-way ANOVA) across the treatments. All

treatments in the absence of IL-1 β showed low GAG degradation and no significant difference in GAG production in comparison with vehicle control (11.8 ± 2.44 % release) (Figure 7.2c, Table 7.3). At the same time in the absence of IL-1 β , prednisolone, S-ethylisothiourrea and low dose quercetin showed a significant difference in GAG degradation in comparison with IL-1 β (alone) treatment (Figure 7.2c). While GAG release values for IL-1 β (23.1 ± 3.39 % release) treated cases were high compared to all other treatments they were not significantly different to vehicle control (Figure 7.2c). Quercetin high dose (in the presence of IL-1 β) treated cartilage showed higher but not significantly different GAG release than IL-1 β alone (Figure 7.2c). No treatment in the presence of IL-1 β showed a significant difference in GAG release in comparison with IL-1 β (alone) treatment (Figure 7.2c).

Table 7.3: Computed concertation (%) of GAG release in three horses.

	CO	CV	PRED	PBZ	EIT	QL	QH	IL	PRED+IL	PBZ+IL	EIT+IL	QL+IL	QH+IL
HORSE 1	3.8	10.1	7.4	12.3	4.5	8.7	15.0	24.3	17.2	15.5	14.9	15.8	33.5
HORSE 2	17.0	10.7	11.3	10.7	10.6	11.9	21.8	19.3	17.6	15.6	23.8	12.0	20.5
HORSE 3	15.9	14.6	7.4	13.7	3.2	11.4	12.7	25.8	8.4	7.6	8.0	18.3	35.0
MEAN	12.2	11.8	8.7	12.2	6.1	10.7	16.5	23.1	14.4	12.9	15.6	15.4	29.7
SD	7.3	2.4	2.3	1.5	3.9	1.7	4.7	3.4	5.2	4.6	7.9	3.2	8.0

7.2.1.3.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1

to 4 (Figure 7.3). Under 10x magnification, qualitative data of GAG degradation determination is presented here (Figure 7.3 a-g). Control, IL-1 β , quercetin high dose (in the presence and absence of IL-1 β) and quercetin low dose (in the presence and absence of IL-1 β) treated cartilage was collected after 5 days explant culture. Fresh, control, quercetin low dose (in the absence of IL-1 β) treated cartilage were graded 1 (Figure 7.3 a, b and c), Quercetin high dose (in the presence and absence of IL-1 β) were graded as 4 (Figure 7.3 d and g). IL-1 β (alone) treated cartilage was graded 3 (Figure 7.3 e) and quercetin low dose (in the presence of IL-1 β) treated cartilage was graded as 2 (Figure 7.3 f) according to OARSI.

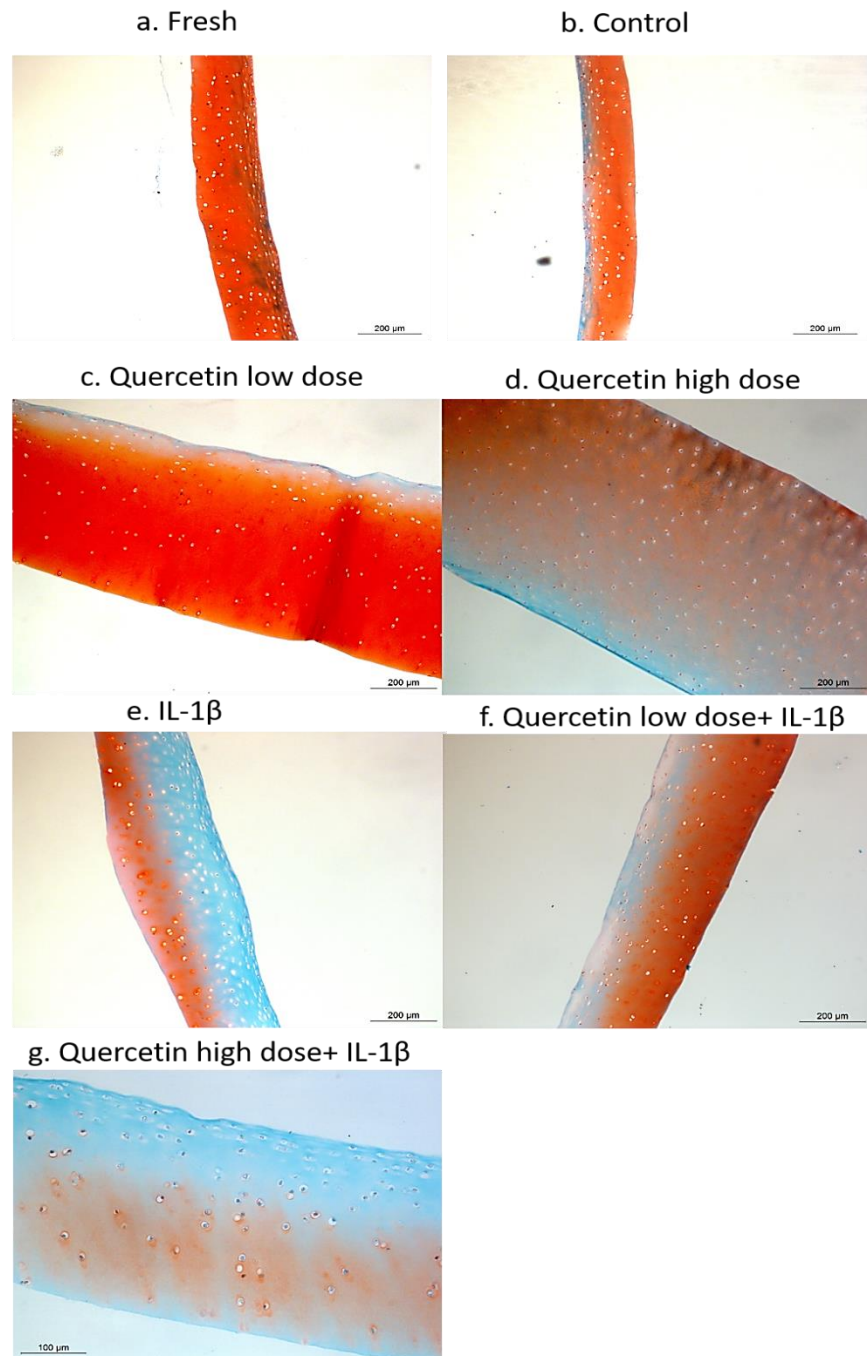


Figure 7.3: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), Quercetin low dose (c), quercetin high (d), IL-1 β (e), quercetin low + IL-1 β (f) and quercetin high + IL-1 β (g). quercetin high does (100 μ g/ml) and quercetin low dose (10 μ g/ml) were used in culture; whereas IL-1 β was used at a concentration of 10 ng/ml. Sections a, b and c were graded as 1, section e was graded as 3, section f was graded as 2 and section d and g were graded as 4 according to OARSI. Magnification x10; scale bars 200 μ m.

7.3 Dose-response of quercetin in an equine cartilage explant model of inflammation associated with osteoarthritis

7.3.1 Result

7.3.1.1 Gross morphology from cartilage explant culture

Cartilage disks were treated with quercetin 300 µg/ml (993 µM), 100 µg/ml (331 µM), 30 µg/ml (99.3 µM), 10 µg/ml (33.1 µM), 1 µg/ml (3.31 µM) in presence and absence of IL-1 β (10 ng/ml), control (medium only) vehicle control (Medium+1% DMSO) and IL-1 β (IL-1 β alone). All cartilage discs showed no visible gross changes during five days of culture, except for those with quercetin 300 µg/ml and 100 µg/ml quercetin (in the presence and absence of IL-1 β) treatments. 300 µg/ml quercetin (in the presence and absence of IL-1 β) treated cartilage disks developed the darkest yellow colour among all treatments. Whereas 100 µg/ml quercetin (in the presence and absence of IL-1 β) showed a lighter yellow colour than 300 µg/ml (in the presence and absence of IL-1 β) (Figures 7.4).

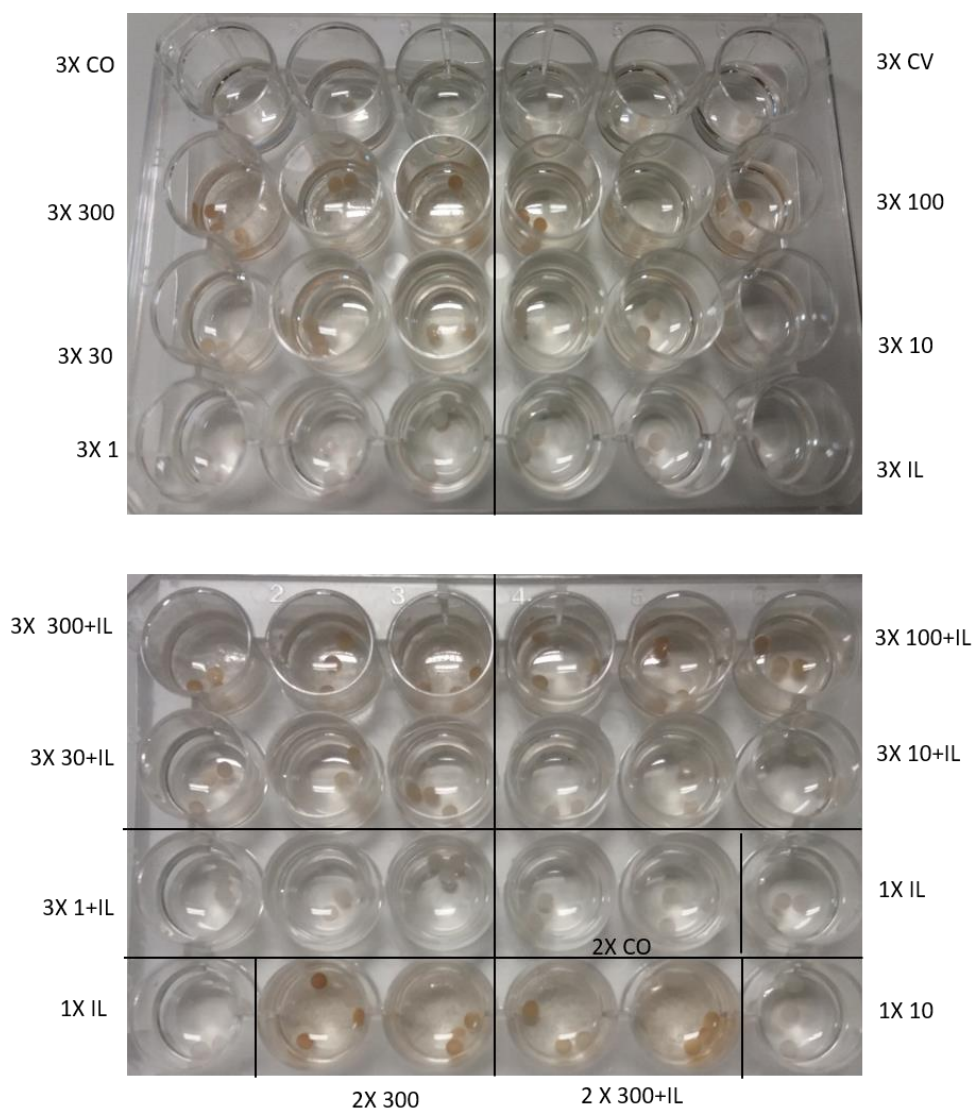


Figure 7.4: Gross morphology of an equine cartilage explant culture (across three 24 well plates) after 5 days treatment with culture medium only (control, CO), medium + 1% DMSO (vehicle control, VC) and interleukin-1 β (10 ng/ml, IL) or quercetin 300 μ g/ml (300), 100 μ g/ml (100), 30 μ g/ml (30), 10 μ g/ml (10), 1 μ g/ml (1) alone or in the presence of IL.

7.3.1.2 Nitric oxide analysis

The calibration curve was developed for nitrite production (Appendix 6, Figure 4). Nitrite production for all treatments, in both

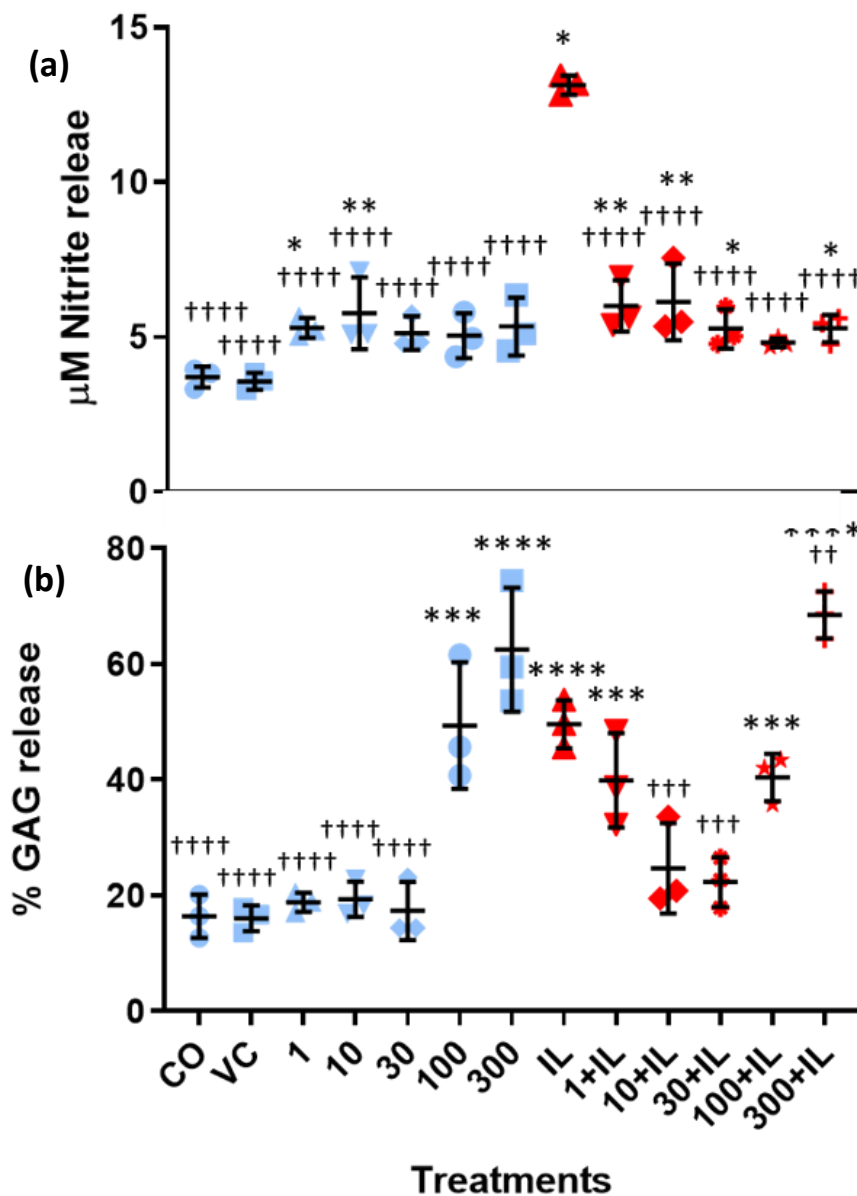


Figure 7.5: Interleukin-1 β (IL)-induced inflammatory response and proteoglycan degradation in equine cartilage explant culture. Explants were exposed for 5 days to IL (10 ng/ml) alone, or quercetin 300 μ g/ml (300), 100 μ g/ml (100), 30 μ g/ml (30), 10 μ g/ml (10), 1 μ g/ml (1), in the presence and absence of IL. Controls were culture media only (CO) and 1% DMSO in culture media (VC). Data are presented as scatter plots with mean and standard deviation. The release of (a) nitric oxide (nitrite) ($n=3$) and (b) glycosaminoglycan (GAG) ($n=3$) were measured and compared using one-way ANOVA with Dunnett's post-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$, significant difference compared to vehicle control; +++ $P < 0.001$ or ++++ $P < 0.0001$, significantly different to IL alone).

Table 7.4: Computed concentration (μM) of nitrite production in three horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	3.96	3.84	5.03	5.08	5.75	5.80	6.36	12.8	5.41	5.50	5.98	4.98	4.77
HORSE 2	3.32	3.29	5.65	7.11	4.79	4.37	5.10	13.4	5.65	5.34	4.78	4.70	5.45
HORSE 3	3.83	3.58	5.20	5.11	4.83	4.96	4.55	13.2	6.95	7.56	5.03	4.77	5.61
MEAN	3.70	3.57	5.29	5.77	5.12	5.04	5.34	13.1	6.01	6.13	5.27	4.82	5.28
SD	0.34	0.28	0.32	1.17	0.54	0.72	0.93	0.31	0.83	1.24	0.63	0.14	0.44

the absence of IL-1 β , were compared with vehicle control and IL-1 β individually. Comparison of nitric oxide production showed there was a significant difference ($P < 0.0001$, F value 34.35, R^2 value 0.9407, one-way ANOVA) across the treatments. Treatments in the absence of IL-1 β showed higher nitric oxide production than vehicle control ($3.57 \pm 0.28 \mu\text{M}$) with 1 $\mu\text{g/ml}$ ($5.29 \pm 0.32 \mu\text{M}$), 10 $\mu\text{g/ml}$ ($5.77 \pm 1.17 \mu\text{M}$) and 100 $\mu\text{g/ml}$ quercetin ($5.04 \pm 0.72 \mu\text{M}$) treated cartilage being significantly different (Figure 7.5a, Table 7.4). All treatments in the absence of IL-1 β showed a significantly lower nitrite production in comparison with IL-1 β alone (Figure 7.5a). The highest nitrite level was detected for IL-1 β treatment ($13.1 \pm 0.31 \mu\text{M}$) (Figure 7.5a). All the treatments in the presence of IL-1 β (except 100 $\mu\text{g/ml}$ quercetin in the presence of IL-1 β , $4.82 \pm 0.15 \mu\text{M}$) showed a significant difference in nitrite production compared to vehicle control (Figure 7.5a). All treatments in the presence of IL-1 β showed a significant difference in nitrite production in comparison with IL-1 β (alone) treatment (Figure 7.5a).

7.3.1.3 GAG analysis

7.3.1.3.1 Colorimetry

Calibration curves of GAG production for three horses are shown in Appendix 6 (Figure 5 a-f). Comparison of GAG % release showed there was a significant difference ($P < 0.0001$, F value 27.57, R^2 value 0.9271, one-way ANOVA) across the treatments. Only 100 $\mu\text{g/ml}$ (22 ± 7.27 % release) and 300 $\mu\text{g/ml}$ quercetin (28.9 ± 3.05 % release) treated cartilage in the absence of IL-1 β showed very high GAG degradation and significant difference in GAG production in comparison with vehicle control (22.6 ± 3.73 % release) but not IL-1 β (alone) (Figure 7.5b, Table 7.5). IL-1 β (alone), 1 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$ and 300 $\mu\text{g/ml}$ quercetin in the presence of IL-1 β showed high GAG degradation which was significantly different to the vehicle control (36.5 ± 13.2 % release, 24.8 ± 13.1 % release, 16.5 ± 0.95 % release, 21.6 ± 4.9 % release, respectively) (Figure 7.5b). At the same time 10 $\mu\text{g/ml}$ and 30 $\mu\text{g/ml}$ quercetin (in the presence of IL-1 β) (30 ± 7.69 % release, 24.6 ± 6.4 % release, respectively) showed lower GAG degradation which was significantly different in GAG release in comparison with IL-1 β (alone) (Figure 7.5b). 300 $\mu\text{g/ml}$ quercetin (in the presence of IL-1 β) showed higher and significantly different GAG degradation than IL-1 β (alone) (Figure 7.5b).

Table 7.5: Computed concertation (%) of GAG release in three horses.

	CO	VC	1	10	30	100	300	IL	1+IL	10+IL	30+IL	100+IL	300+IL
HORSE 1	20.2	17.9	18.9	18.4	14.5	61.7	74.4	53.8	48.6	33.6	26.5	42.0	72.5
HORSE 2	12.7	16.7	17.1	22.8	23.2	40.8	53.6	45.5	38.8	19.5	17.9	43.4	68.7
HORSE 3	16.5	13.6	20.5	16.9	14.4	45.7	59.5	49.6	32.3	20.9	22.7	35.8	64.4
MEAN	16.4	16.1	18.8	19.4	17.4	49.4	62.5	49.6	39.9	24.7	22.3	40.4	68.5
SD	3.72	2.21	1.67	3.08	5.04	10.9	10.7	4.15	8.17	7.80	4.31	4.08	4.06

7.3.1.3.2 Histology

A qualitative study of GAG breakdown was observed by safranin O and fast green (SOFG) staining characteristics ranging from grade 1 to 4 (Figure 7.6). Under 10x magnification, qualitative data of GAG degradation determination is presented here (Figure 7.6 a-i). Fresh cartilage was graded as 0 (Figure 7.6 a). Control, 1 µg/ml quercetin (in the absence of IL-1 β) and 10 µg/ml quercetin (in the absence of IL-1 β) were graded as 1 (Figure 7.6 b, c and d). 300 µg/ml quercetin (in the presence and absence of IL-1 β) and IL-1 β (alone) treated cartilage were graded as 4. 1 µg/ml quercetin (in the presence of IL-1 β) treated cartilage was graded as 3 (Figure 7.6 g). The 10 µg/ml quercetin (in the presence of IL-1 β) treated cartilage was graded as 2 (Figure 7.6 h) according to OARSI.

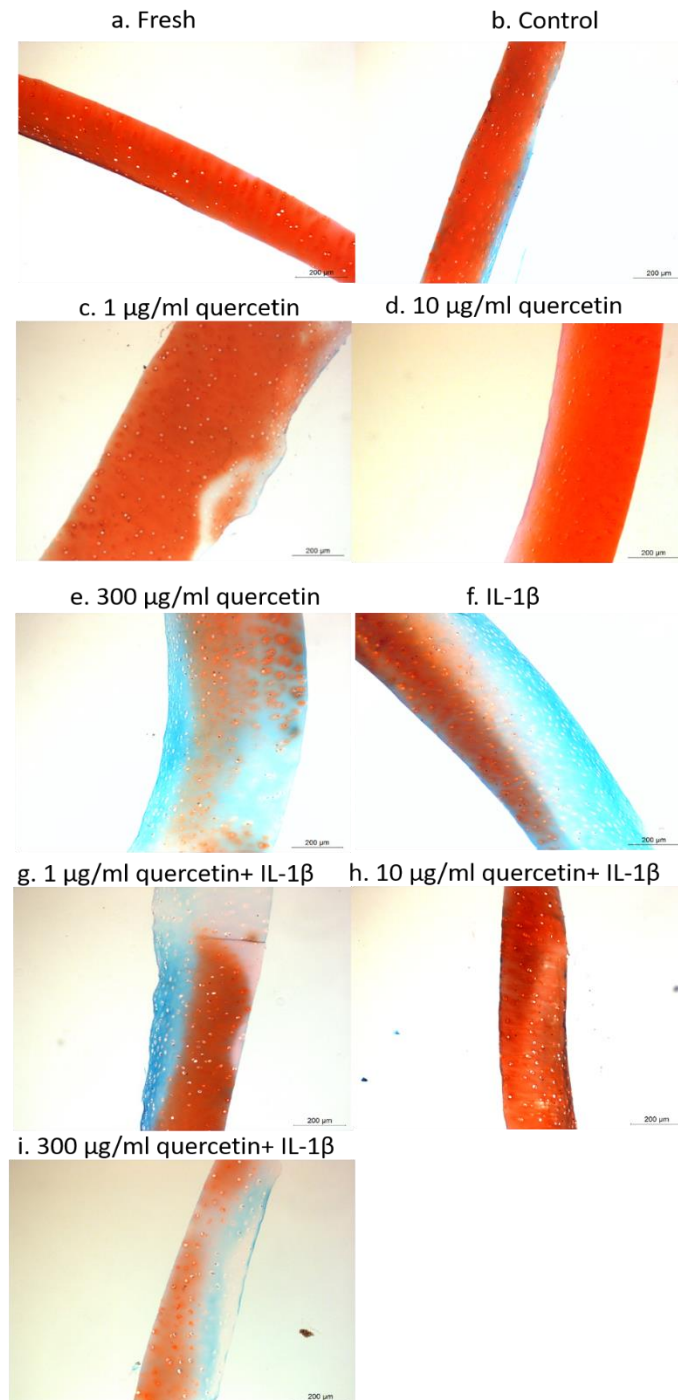


Figure 7.6: Qualitative study of GAG degradation visualized by staining with Safranin O and Fast green of (a) fresh equine cartilage and (b-g) explant cultures. Fresh cartilage was collected immediately after sample collection. Explants were stimulated for 5 days with culture medium only (CO) (b), 1µg/ml quercetin (c), 10 µg/ml quercetin (d), 300 µg/ml quercetin (e), IL 1β (f), 1µg/ml + IL 1β (g), 10 µg/ml + IL 1β (h) and 300 µg/ml + IL 1β (i); IL 1β was used at a concentration of 10 ng/ml. Section a was graded as 0. Sections b, c and d were graded as 1, section e, f and i were graded as 4 according to OARSI. Section g was graded as 3 and section h was graded as 2 according to OARSI. Magnification x10; scale bars 200 µm.

7.4 Discussion

Phenylbutazone, prednisolone, and S-ethylisothiourrea found similar inhibitory effects on nitric oxide and GAG as monitored in Chapter 4. In this chapter, quercetin significantly inhibited nitric oxide production (92-100%) in the presence of IL-1 β at a concentration range of 1-300 $\mu\text{g/ml}$ and therefore no IC₅₀ could be determined. In a previous study, it was also observed that quercetin (1.51-30.2 $\mu\text{g/ml}$) could inhibit iNOS in a dose-dependent manner which involved blockade of NF- κ B activation and pro-inflammatory genes in the human hepatocyte-derived cell line termed Chang liver (CHL) (García-Mediavilla *et al.*, 2007). Quercetin has been shown in a mouse macrophage cell line (RAW 264.7) to inhibit LPS-induced nitric oxide production by inhibiting iNOS and cyclooxygenase-2 (COX-2) (gene expression), but not enzyme activity, in a concentration-dependent manner (1.51-12.9 $\mu\text{g/ml}$) without cytotoxic effect (Chen *et al.*, 2001).

Similar to previous observations, a significant difference of PGE₂ production was not observed between vehicle control and IL-1 β and it is hypothesized that this is due to too high concentration of DMSO as vehicle as discussed in previous chapters. In a literature study, it was observed that quercetin (30.3 $\mu\text{g/ml}$), at 0.1% DMSO was able to inhibit PGE₂ production in the presence of IL-1 in rheumatoid

synovial fibroblasts cell culture (RASFs) (Sung *et al.*, 2012). Moreover in another previous study it was observed that quercetin (15.1 $\mu\text{g/ml}$) was able to inhibit cytokine mixture induced (mixture of IL-1 β , TNF α , IFN α (20 ng/ml each) PGE₂ production also in the presence of 0.1% DMSO in a culture using human alveolar epithelial A549 cells (Banerjee *et al.*, 2002). These two publications strongly support the hypothesis that that 1% DMSO is too high a concentration for the stimulation of PGE₂.

In the initial study, a high dose of quercetin showed higher GAG breakdown in the absence of IL-1 β than IL-1 β alone which raised the question whether this concentration of quercetin used (100 $\mu\text{g/ml}$) was stimulating GAG breakdown. The inhibitory effect on GAG breakdown by quercetin has been observed to lesser extent following inhibition of the expression of MMP3 in bovine chondrocyte culture (Jackson *et al.* 2006). It has been observed that in retinoic acid or interleukin IL-1 β stimulated bovine articular cartilage explant cultures, quercetin protected cartilage from stimulated aggrecan loss in a dose-dependent manner (3.02-15.11 $\mu\text{g/ml}$) and also suppressed gene expression of ADAMTS-4 and ADAMTS-5 (Lay *et al.*, 2012). In the present study, it was observed that quercetin effectively inhibited GAG breakdown in a dose dependent manner up to 30 $\mu\text{g/ml}$. However at doses greater than 30 $\mu\text{g/ml}$ quercetin stimulated of GAG breakdown.

7.5 Conclusion

In this chapter, quercetin was observed as a potent nitric oxide inhibitor irrespective of concentration used here. The complex inhibitory effect on GAG breakdown by different concentrations of quercetin might be an indication that it has a small therapeutic window (10-30 µg/ml) to protect cartilage degradation. Cell viability was not determined for quercetin in cartilage explant model in this study. Though the inhibitory effect of quercetin on PGE₂ production was obscured by 1% DMSO, quercetin was found to inhibit PGE₂ production in previous studies. Further studies on inhibitory effect of quercetin on ADAMTS-4, ADAMTS-5 and MMPs will help to understand its potential as a DMOAD.

Chapter 8

General discussion

Solubility has a decisive role in the success of a drug candidate. The low solubility of a drug causes problems for *in vitro* and *in vivo* assays and also interferes with drug development (Di *et al.*, 2012). In a cell-based system, the required drug solubility in an aqueous environment may not be achieved and therefore a co-solvent is used to solubilize. However, co-solvents can alter drug permeability into tissue and it is essential to choose the most appropriate co-solvent according to the purpose of the study as well as the physicochemical property of the compounds to be tested (Sugano *et al.*, 2001). Co-solvents (e.g. DMSO, absolute ethanol, acetone, 55% polyethylene glycol-400+45% ethanol mixture (PEM), etc.) are typically used in cell culture for the poorly water-soluble drugs.

DMSO is a commonly used co-solvent in cell culture and the effect of DMSO varies not only due to cell type but also species and age of the animals (Nixon *et al.*, 1992; Platt *et al.*, 1998). It has been observed in equine cartilage explant culture that DMSO at $\geq 5\%$ concentration suppressed matrix metabolism and proteoglycan synthesis without causing chondrocyte death (Smith *et al.*, 2000). In the present study 1% DMSO has a negligible effect on GAG degradation, nitric oxide production, and cell viability. However, 1% DMSO did affect PGE₂ production but the mechanism is not clear. In a previous study, it has been observed that DMSO at the concentration range of 0 -1% did not show any effect on COX-1

dependent PGE₂ production in unstimulated (in absence IL-1 β) Caco-2 cells (Hollebeeck *et al.*, 2011). However, a close examination of this data showed that DMSO was stimulating PGE₂ production in unstimulated Caco-2 cells which mimics the high PGE₂ production in vehicle control (medium+ 1% DMSO) observed in the present study. Moreover, it has also been observed that COX-2 dependent PGE₂ production from IL-1 β was suppressed by DMSO (1%) in Caco-2 cells (Hollebeeck *et al.*, 2011). DMSO at concentrations $\leq 1\%$ have been recommended by Ingels *et al.* (2004) to use in standard screening *in vitro* procedures to study drug transports and did not show any cytotoxicity in Caco-2 cells at that concentration (Hollebeeck *et al.*, 2011, Ingels *et al.*, 2004).

In order to determine PGE₂ concentration, a significant amount (150 μ l) supernatant was taken from a total of 600 μ l of cell culture medium which restricts the opportunity to detect other OA-modulators (e.g. MMPs, ADAMTS, Collagen, COX, etc.). Observing effects on these OA-modulators by testing nutraceuticals will help to understand their beneficial effects on OA like conditions.

It has been demonstrated that a direct and quantifiable relationship is present between chondrocyte death and loss of proteoglycans as chondrocyte death plays a definite role in the initiation of cartilage degeneration in post-traumatic OA (Huser *et al.*, 2006). In support

of this finding, they also suggested that the apoptotic-like death of chondrocytes releases degenerative enzymes, either directly into the surrounding extracellular matrix, or via the production of apoptotic bodies which carry the degradative enzymes. Moreover, the loss of chondrocytes through apoptosis may increase matrix catabolism through a local reduction in protease inhibitor production (Huser *et al.*, 2006). There is also evidence that ECM breakdown may lead to cell death as the occurrence of 'anchorage dependence' states that cells need to be attached to the ECM or to each other for survival, function, and growth; otherwise, they are subjected to apoptosis. GAG degradation and disruption of the collagen network led to cartilage degradation and this may cause the disturbance of chondrocyte anchorage to the ECM and ultimately cell death (Aigner and Kim, 2002). However, in Chapter 4, it has been observed that IL-1 β (alone) caused significantly high GAG degradation but no significant difference in cell viability while comparing with fresh and control cultured cartilage. A similar feature has also been observed for resveratrol (100 μ g/ml) in the absence of IL-1 β . It is not clear from this study whether cell death leads to GAG breakdown as there was no observed dynamic range in the chondrocyte viability. However, it is clear that GAG breakdown does not initiate cell death at least in the early stages of OA which is contrary to Aigner and Kim (2002). This is supported by Jeffrey *et al.* (1995) where they showed in bovine articular cartilage that the viability of chondrocytes was

surprisingly irrepressible when tissue disruption is severe. Also, it is well known that chondrocytes remain alive independent of the extracellular matrix in primary chondrocyte culture.

In the herein study, it has been observed that IL-1 β (alone) induced high NO production in cartilage without significant cell mortality. At the same time, decreased cell viability was observed with low NO production in the case of resveratrol in the presence of IL-1 β (100 μ g/ml). These findings suggest that NO has little role in chondrocyte viability at the early stages of OA. It has been observed in the literature that NO production increased when chondrocytes were stimulated by endogenous sources (IL-1, TNF α , IFN γ , and LPS alone or in a combination) without any alteration in chondrocyte viability or apoptotic change consistent with the herein observations (Blanco *et al.*, 1995; Carlo *et al.*, 2002).

It has been observed in bovine chondrocytes and human articular cartilage that endogenous NO produced by stimulated chondrocytes is a key molecule to activate metalloproteinases which caused inflammation-induced cartilage catabolism (Murrell *et al.*, 1995). Moreover, it has been observed that endogenously produced NO is a major mediator to suppress proteoglycan synthesis in rabbit articular cartilage (Taskiran *et al.*, 1994). Differing from all of these findings, it has been observed in this study that resveratrol and

quercetin at 100-300 µg/ml (in the presence and absence of IL-1 β) effectively inhibited NO production but failed to inhibit GAG degradation. Rather it has been observed that even in unstimulated cartilage, resveratrol and quercetin (100-300 µg/ml) caused a very high GAG degradation with minimal NO production which contradicts GAG degradation by NO. This study suggests that NO is not a key mediator of GAG degradation in the OA-like condition.

To discover a DMOAD, it is important to understand the relationship between different endpoints of OA at different stages of disease progression. Understanding the interaction of different endpoints like cartilage synthesis, collagen degradation, expression and activity of MMPs and ADAMTS with GAG degradation and cell death could be an effective paradigm for optimising the efficacy of a potential DMOAD.

In this study, the effects of resveratrol, piceatannol, and quercetin have been observed in OA like conditions in equine cartilage explant culture. Piceatannol and quercetin (1-300 µg/ml) showed a wider inhibitory effect on NO production than that of resveratrol (10-300 µg/ml). The inhibitory effect on GAG degradation by piceatannol (10-300 µg/ml) was wider than those of resveratrol and quercetin (10-30 µg/ml). In stimulated cartilage, at 100 µg/ml concentrations cell viability decreased for resveratrol while piceatannol showed no

Table 8.1: Comparison of anti-inflammatory properties and pharmacokinetics between resveratrol, piceatannol and quercetin in from this and previous studies.

Target	Function	Resveratrol	Piceatannol	Quercetin
NF κ B	Transcription	Suppressor ¹	Suppressor ¹⁸	Suppressor ² ₈
HIF	Transcription	HIF-1 α ³ HIF-2 α ⁸ Suppressor	HIF-1 α ²³ Suppressor	HIF-1 α HIF-2 α Inducer ³¹
COX-1 IC ₅₀ (μ M)	Prostaglandin	0.535 ⁷ , 2.27 ⁴	4.71 ⁷	2.91 ³⁴
COX-2 IC ₅₀ (μ M)	Prostaglandin	0.996 ⁷ , 3.40 ⁴	0.01 ⁷	10.5 ³⁴
iNOS	Nitric Oxide	Suppressor ²	Suppressor ⁹	Suppressor ² ₇
MMP13	Collagenase	Suppressor ⁵	Suppressor ²⁴	Suppressor ³ ₂
ADAMTS-4 IC ₅₀ (μ M)	Aggrecanase	Suppressor ⁶ 50 ¹⁷	Suppressor 1 ²¹ K _i = 2.68 μ M ²²	Suppressor ⁶ _{,30}
ADAMTS-5 K _i (μ M)	Aggrecanase	Suppressor ¹⁴	Suppressor K _i = 1.18 ²²	Suppressor ³ ₀
SIRT1	Apoptosis inhibition	Inducer ¹	Inducer ¹³	Inducer ²⁹
5-LOX IC ₅₀ (μ M)	Pain	1.02 ¹⁰ , >50 ⁴	0.76 ⁴	3.29 ⁴ , 2.07 ³⁵
NO production IC ₅₀ (μ M)	Inhibition	4.38 - 43.8	2.5	0 - 3.31
GAG breakdown IC ₅₀ (μ M)	Inhibition	43.8 - 438	8.4	33.1 - 99.3
T _{1/2} (Hour) (Rat)	Plasma half life	0.75 ¹⁵ , 0.59 ¹⁶ 9.2 ¹¹ (oral)	5.21 (IV) ²⁵ 3 (Oral) ²⁵	0.35 ³³
CL (IV) (l/ h/kg)	Plasma clearance	1.68 ¹⁶	2.13 ²⁶ , 1.986 ²⁵	---
Bio- availabaility (Oral) (%)		<1% ^{12,11}	50.7 $\pm$ 15.0 ²⁵	No parent drug detected ³³

¹Bagul *et al.*, 2015; ²Cichocki *et al.*, 2008; ³Kim *et al.*, 2013; ⁴Kutil *et al.*, 2015; ⁵Lei *et al.*, 2008; ⁶Mabrouk *et al.*, 2006; ⁷Murias *et al.*, 2004; ⁸Qin *et al.*, 2017; ⁹Zhang *et al.*, 2014; ¹⁰Kim *et al.*, 1999; ¹¹Walle *et al.*, 2004; ¹²Wenzel and Somoza, 2005; ¹³Hong *et al.*, 2012; ¹⁴Kang *et al.*, 2018; ¹⁵Regev-Shoshani *et al.*, 2003; ¹⁶Vijayakumar *et al.*, 2016; ¹⁷Cudic *et al.*, 2009; ¹⁸Ashikawa *et al.*, 2002; ¹⁹Islam *et al.*, 2004; ²⁰Kutil *et al.*, 2014; ²¹Lauer-fields *et al.*, 2009; ²²Cudic *et al.*, 2010; ²³Song *et al.*, 2015; ²⁴Tang *et al.*, 2017; ²⁵Lin *et al.*, 2010; ²⁶Roupe *et al.*, 2006; ²⁷Chen *et al.*, 2001; ²⁸Cho *et al.*, 2003; ²⁹Hung *et al.*, 2015; ³⁰Lay *et al.*, 2012; ³¹Park *et al.*, 2008; ³²Qiu *et al.*, 2018; ³³Yang *et al.*, 2005; ³⁴Mutoh *et al.*, 2000; ³⁵Doucet *et al.*, 2018.

alteration (cell viability in quercetin was not determined). Comparing the inhibitory effects on cell viability, NO production and GAG degradation, piceatannol showed a wider inhibitory window than that of resveratrol and quercetin (Table 8.1). In the literature, it has been observed that piceatannol shows superior pharmacokinetics and activity with specific OA modulators (i.e. COX-2, ADAMTS4, ADAMTS-5, 5-LOX and bioavailability) than resveratrol and quercetin (Table 8.1). Moreover, resveratrol (100-300 µg/ml) and quercetin (300 µg/ml) showed a high GAG breakdown even in unstimulated cartilage which may need to be taken under consideration as a potential DMOAD candidate. At the same time, piceatannol showed an interesting feature in GAG breakdown in unstimulated cartilage which may be an indication of basal GAG synthesis. Further studies on GAG synthesis and cell viability in healthy cartilage can be an option to identify the effects of piceatannol to discover its potential to improve cartilage physiology. The bioavailability of these nutraceuticals can be improved by microencapsulation as it has been shown by Watts *et al.* (2016) for resveratrol. Considering the inhibitory effects and pharmacokinetics, piceatannol may have a better prospect as a DMOAD than that of resveratrol and quercetin.

Table 8.2: Comparison of anti-inflammatory properties and pharmaco-kinetics between GLPG1972 and piceatannol.

Target	Species	GLPG1972	Piceatannol
GAG (IC₅₀)	Human	<1 μM (Aggrecan fragment) ²	---
	Mouse	2 μM^2	---
	Horse	---	8.4 μM
ADAMTS-5 (IC₅₀)	Human	20 nM ²	---
	Rat	27 nM ²	---
	collagen-model (FRET) substrates	---	K _i = 1.18 μM (competitive inhibitor) ^{4,6}
ADAMTS-4 (IC₅₀)	Human	57 nM ³	---
	collagen-model (FRET) substrates	---	1 μM^5
5-LOX (IC₅₀)	Enzyme assay	---	0.76 μM^5
NO (IC₅₀)	Horse	---	2.5 μM
Cell viability	Horse	-	78.8 $\pm$ 3.57 %
T_{max} (Oral) (h)	Human	1-4 ¹	---
	Rat	---	0.67-2 ⁶
T_{1/2} (h)	Human	10 (Oral) ¹	---
	Rat	---	3 (Oral) 4.23 (IV) ⁶
Excretion in urine (unchanged)	Human	<11%(upto 24 h post dosing) ¹	
	Rat	---	11% (upto 84 h post dosing) ⁶
Bioavailability	Rat	---	50.7 $\pm$ 15.0% ⁶

¹van der Aar *et al.*, 2018; ²Clement-Lacroix *et al.*, 2018; ³Clement-Lacroix, *et al.*, 2017b; ⁴Cudic *et al.*, 2009b; ⁵Kutil *et al.*, 2015; ⁶Lauer-Fields *et al.*, 2008; ⁷Lin *et al.*, 2010; ^{28e}Roupe, *et al.*, 2006.

Recent reports have revealed that the Galapagos pharmaceutical company introduced a new DMOAD (GLPG1972) in human clinical trials. Piceatannol and GLPG1972 have been compared within Table 8.2. GLPG1972, a selective ADAMTS-5 inhibitor, has shown anti-catabolic activity in cartilage explants with moderate ADAMTS-4 inhibition (Clement-Lacroix, *et al.*, 2017; Clement-Lacroix, *et al.*, 2017a). Moreover, in a DMM model, it was observed that GLPG1972 effectively reduced proteoglycan loss, cartilage damage score, cartilage fibrillation and increased the subchondral bone thickness (Clement-Lacroix *et al.*, 2017; Lepescheux *et al.*, 2019). GLPG1972 showed a remarkable decrease in serum AGRS (Alanine-Arginine-Glycine-Serine) both in diseased and healthy human trial patients (Deckx *et al.*, 2019; van der Aar *et al.*, 2018). In the diseased human trial, GLPG1972 increased drug-related transaminase level with mild and transient adverse effects (Deckx *et al.*, 2019). It has been claimed that GLPG1972 did not show any adverse effect on the heart in rat and human (Deckx *et al.*, 2019; van der Aar *et al.*, 2018). GLPG1972 has , for chondrocyte viability and MMP inhibition but no data was reported (Clement-Lacroix *et al.*, 2018; Clement-Lacroix *et al.*, 2017). On the other hand, piceatannol, a competitive ADAMTS-5 inhibitor (Cudic *et al.*, 2009b), showed potent inhibition of GAG degradation and, nitric oxide production with no alteration in cell viability. Moreover, it has been reported that piceatannol is a potent inhibitor of 5-lipoxygenase enzyme which is responsible for

pain in OA (Kutil *et al.*, 2014). In addition, piceatannol is a potent inhibitor of ADAMTS-4, MMP1, MMP2, MMP8, MMP9, MMP12 and MMP13 (Cudic *et al.*, 2009; Lee *et al.*, 2009; Maruki-Uchida *et al.*, 2013; Tang *et al.*, 2017; Lauer-Fields *et al.*, 2008).

It has also been reported that piceatannol can promote collagen synthesis (Matsui *et al.*, 2010b) and induction of osteoblast's maturation and differentiation, followed by an increase in bone mass (Chang *et al.*, 2006). Piceatannol has been observed to have good oral absorption and be eliminated predominantly via non-urinary routes. It was also observed that piceatannol was highly distributed into tissues and highly extracted by the liver (Roupe *et al.*, 2006). Moreover, orally induced piceatannol has 3 hours half-life in the rat (Lin *et al.*, 2010) which would extrapolate to 12 hours in human and makes it a potential drug candidate. Piceatannol, a small molecule nutraceutical, can become a superior DMOAD candidate by optimising its potency, selectivity and pharmacokinetics through synthetic chemistry. From this study and literature reviews, it has been observed that the major features associated with OA progression (Chondrocyte viability, inhibition of GAG degradation, ADAMTS-4, ADAMTS-5, MMP 9, MMP 13 and promoting collagen synthesis) could potentially be provided by piceatannol as a DMOAD.

As a future study, the inhibitory effect of piceatannol, resveratrol and quercetin on collagen breakdown could be observed by ELISA, electrophoresis and immunoblot analyses, immunohistochemistry in cartilage explant culture. Moreover, the effects of these phenols on collagen synthesis by immunohistochemistry, mRNA expression by PCR in cartilage explant culture can also be an important parameter to understand their prospects in cartilage repair. In addition, their effects on GAG synthesis can be measured by radiolabelled GAGs and this may help to get an answer as to whether piceatannol is actually maintaining basal GAG or not. Understanding their inhibitory effects on the expression and activity on MMP13, ADAMTS-4 and ADAMTS-5 may give a hint of how potent they are in situ. These studies may help to answer whether these nutraceuticals are inhibiting cartilage destruction only or also helping in cartilage repair/regeneration without any cytotoxic effect. Besides these, a study on different routes of administration and bioavailability of these nutraceuticals may help to interpret their prospect for human use. Further investigation using an in vivo DMM model and population trial may help to understand the future prospect of these phenols as a DMOAD candidate.

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Appendices

10.1 Appendix 1

A. Processing Protocol

1. Collect samples in 10% Neutral Buffered Formalin and fix for a 24 hrs minimum
2. Write down each sample in the log book as the year and consecutive number e.g. 07-001, and then write the number on the corresponding cassette containing the sample.
3. Place cassettes into 70% ethanol until ready to use
4. When ready place samples in 70% ethanol basket in the tissue processor
5. Press ethanol to lower lid and submerge the cassettes in the 70% ethanol
6. Press start
7. Enter programme number 1
8. Set date to start- 0 = today, 1= tomorrow
9. Set start time- usually 4 pm so ready at 9 am the following day
10. Press start

B. Embedding protocol

1. Remove cassettes from the processor and tip them into the melted wax in the embedding machine
2. Make sure cold plate is switched on
3. Take cassette out of the hot wax, open it and place the sample into a metal mould
4. Fill the mould with wax, place onto a cold plate and hold the sample in the desired position with forceps until the wax begins to set
5. Top up the mould with wax and put the labelled cassette on top and leave on a cold plate to set

6. Once set, remove from the mould, trim off excess wax and store in the fridge until ready to cut
7. Put moulds into histoclear overnight to de-wax them

C. Microtoming protocol

1. Switch on a microtome, water bath (37°C) and hot plate (40°C)
2. Remove the wax block from the fridge and carefully trim off excess wax around the sample to leave clean cut edges
3. Put block into the holder on the microtome
4. Insert the trimming blade (kept on the left side) and lock in place
5. Line up the block with the blade and lock in place
6. Set machine to TRIM (30 microns)
7. Depress foot pedal to begin trimming, trim until the sample is reached
8. Remove trimming blade and replace with a cutting blade (kept on the right side)
9. Set machine to SECTION (5 microns at speed 3)
10. Cut sections
11. Lift sections off the machine with soft brushes and place onto a glass tile
12. Pipette 30% ethanol under the sections to flatten them
13. Trim sections to separate then float them off the tile into the water bath until they are flat
14. Label slides with cassette number then use it to pick up the sections
15. Put slide onto the hot plate and leave until water has evaporated
16. Place into a slide rack and put into an oven at 44° C overnight

D.1 H&E Staining Protocol

1. Place slides into histoclear for 30 mins to dewax
2. Drain on a paper towel
3. 2 mins in 100% ethanol
4. 2 mins in 95% ethanol
5. 2 mins in 70% ethanol
6. Wash under running water in a glass jar
7. 4 mins in Haemotoxylin
8. Wash in glass jars until water runs clear
9. 1 dip in 1% Acid IMS
10. Wash in water
11. 2 dips in ammoniated water
12. Check staining and repeat if necessary
13. Wash in water
14. 10 mins in Eosin
15. Wash in glass jars until water runs clear
16. Dry on a paper towel
17. 6 dips in 70% ethanol
18. 6 dips in 95% ethanol
19. 6 dips in 100% ethanol
20. Dab to remove excess
21. 5 mins in Histoclear
22. 5 mins in Xylene in the fume hood
23. WEAR DOUBLE GLOVES-take a slide from the xylene and pipette DPX onto the sample

24. Place a coverslip over the sample and push down with a pipette tip to remove air bubbles

25. Leave to dry in fume hood

D.2 SOFG staining protocol

Ingredient	Staining time
0.1% Fast Green	3 min
1% Acetic Acid Water	2 dips
0.1% Safranin O	4 min
70% Ethanol	1 dip
95% Ethanol	3 dips
100% Ethanol	2 Times -2 min each time
50/50 Ethanol / Xylene	2 min
Xylene	3 Times - 3 min each Time

Stain Preparation

- Safranin O Stock Solution 1% Solution
- Take 5 g Safranin O (C.I.50240) for 500 ml of dH₂O
- Working Solution for pH 5.2
- Take 30 ml of 1% Safranin- O Stock Solution and 270 ml of dH₂O
- Yields 300 ml Of 0.1Safranin-O pH 5.2
- Fast Green Stock Solution 1%
- 5g Fast Green for 500 ml of dH₂O
- Staining Solution 0.1% Fast Green
- Take 30 ml Fast Green Stock Solution and 270 ml of dH₂O

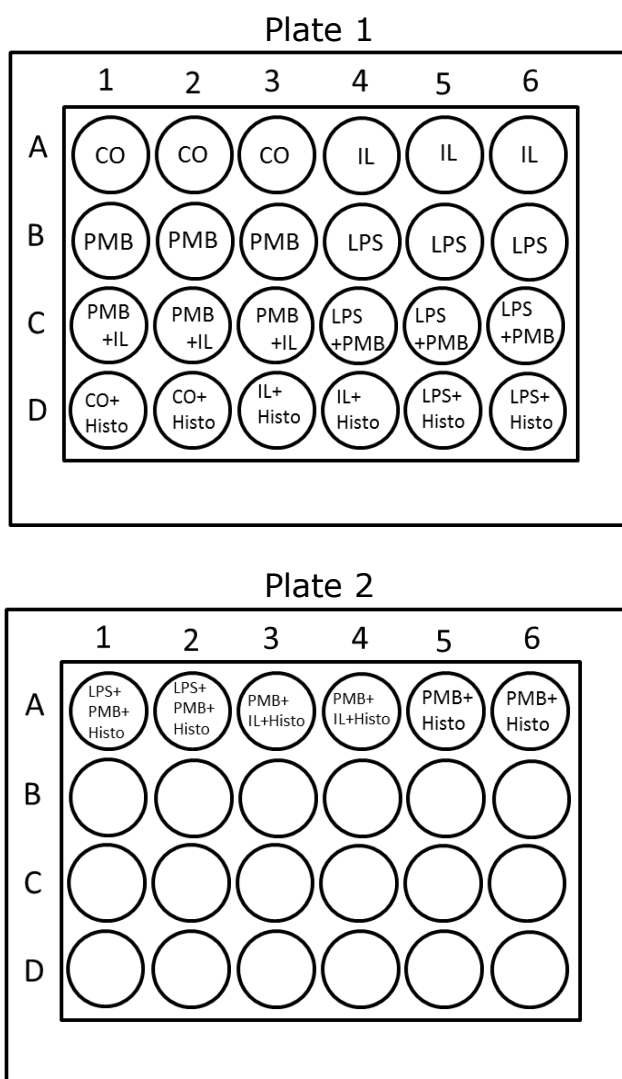


Figure 1a: Plate design for explant culture for study on validation of inflammatory stimulant and vehicle in equine cartilage explant model. The design was repeated for each horse. For treatment abbreviations, refer to Table E1.

* Histo= cartilages cultured for histological slide preparation.

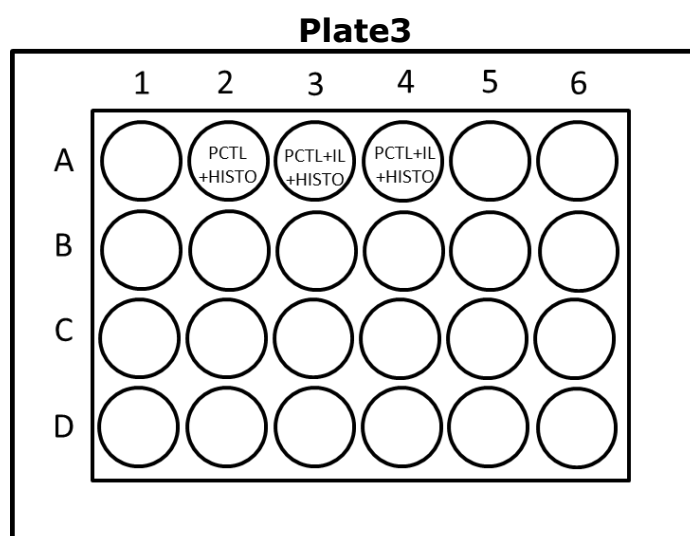
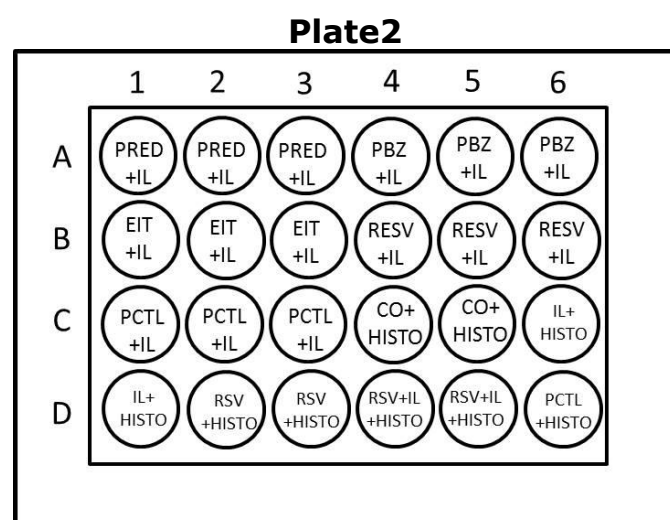
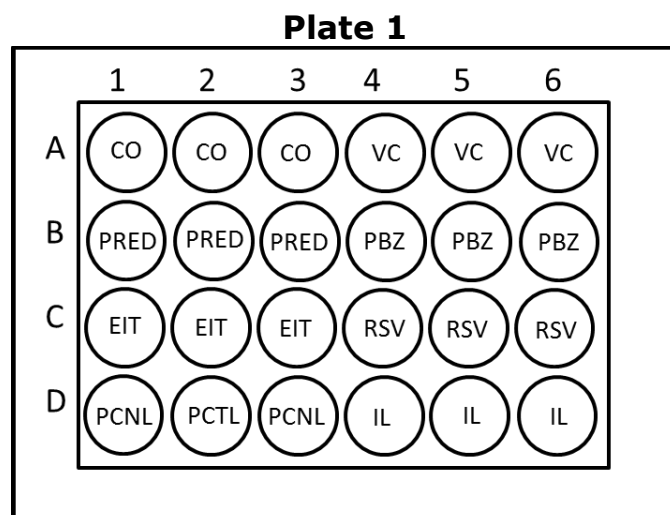


Figure 1b: Plate design for explant culture for study on effects of resveratrol and piceatannol in an equine explant cartilage model of inflammation associated with osteoarthritis. For treatment abbreviations, refer to Table E1. The design was repeated for each horse. * Histo= cartilages cultured for histological slide preparation).

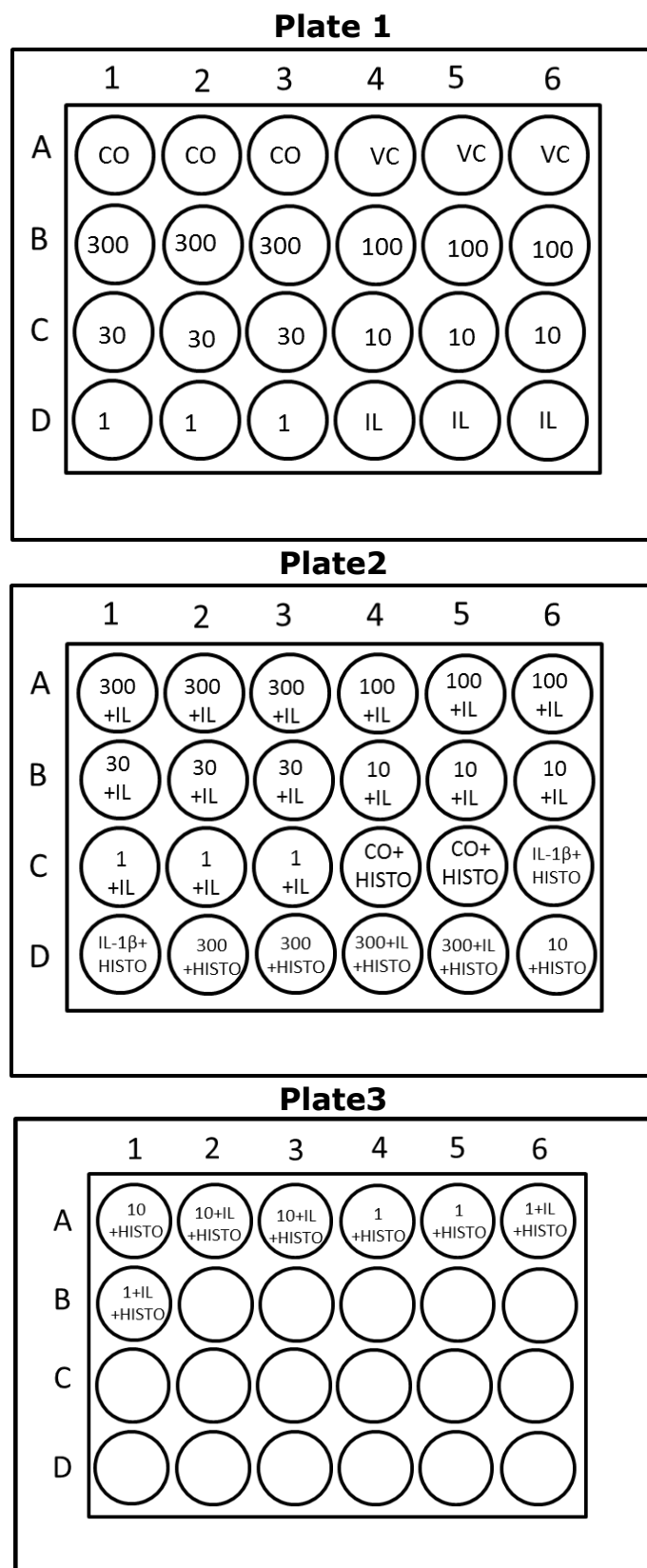


Figure 1c: Plate design for explant culture for dose response studies of resveratrol, piceatannol and quercetin. The design was repeated for each horse. For treatment abbreviations, refer to Table E1. * Histo= cartilages cultured for histological slide preparation).

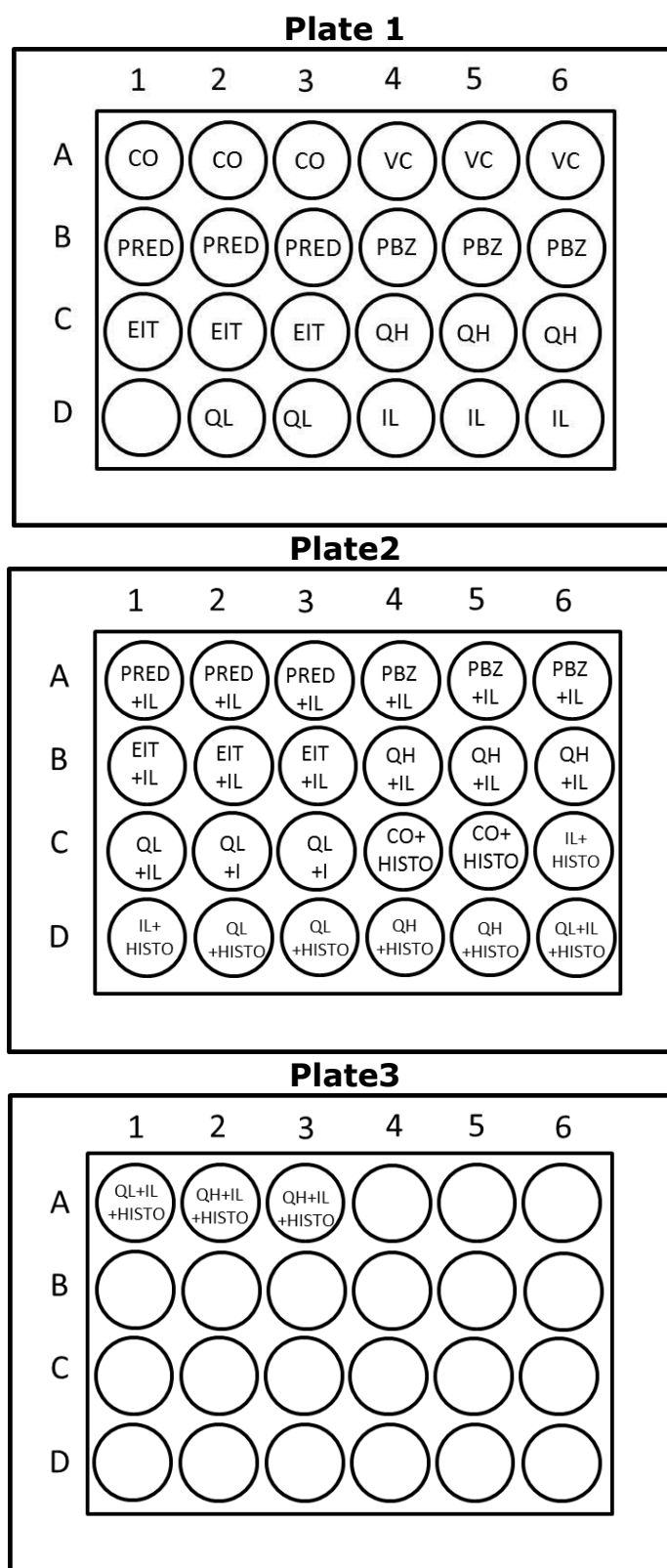


Figure 1d: Plate design for explant culture for study on comparative study on effect of quercetin in an equine cartilage explant model of inflammation associated with osteoarthritis. The design was repeated for each horse. For treatment abbreviations, refer to Table E1. * Histo= cartilages cultured for histological slide preparation).

10.2 Appendix 2

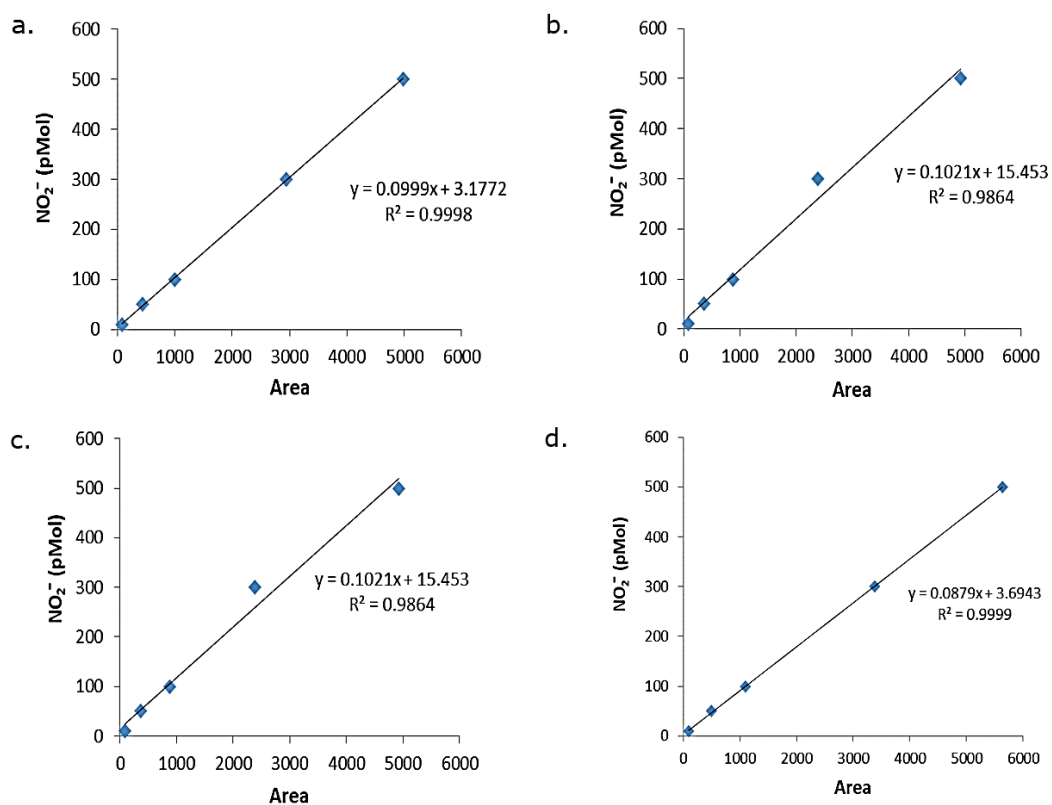


Figure 1 (a-d): Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horse 1 (a), horse 2 (b), horse 3 (c) and horse 4 (d)

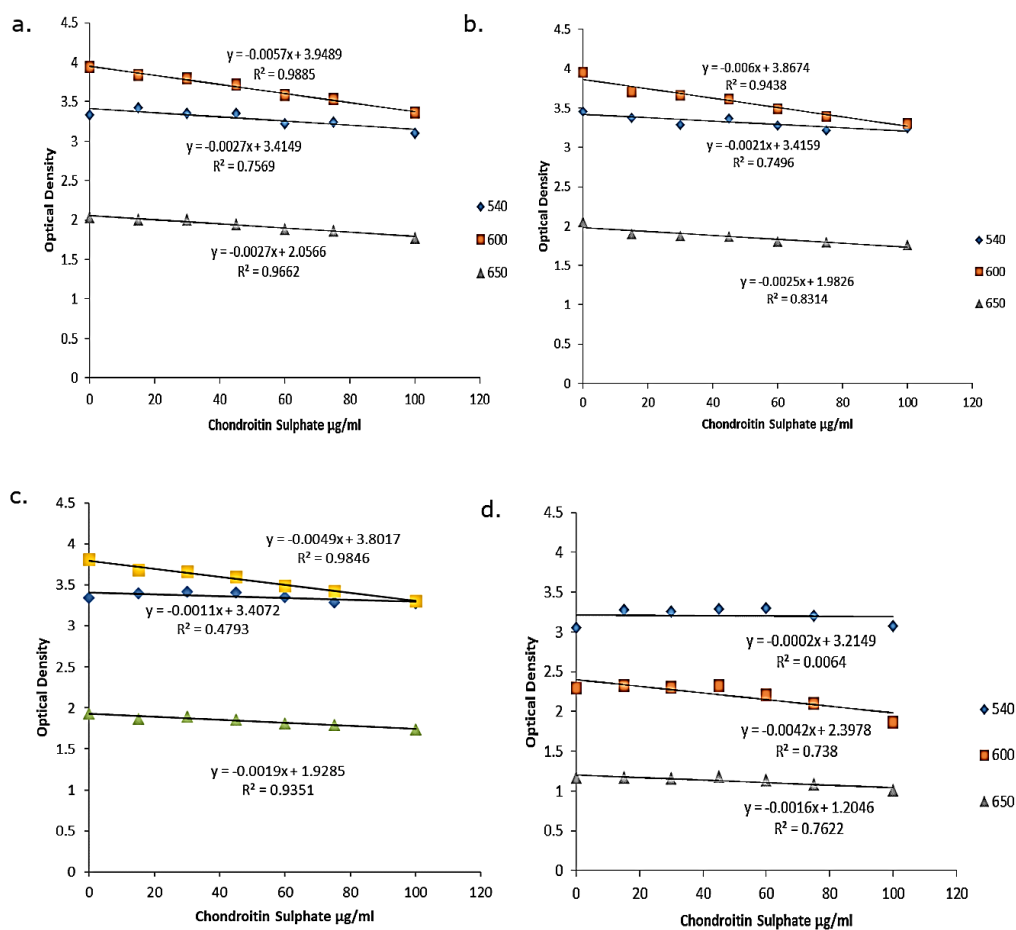


Figure 2 (a-d): Glycosaminoglycan (GAG) calibration curve. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for in horse 1 supernatant and cartilage (a), horse 2 supernatant and cartilage (b), horse 3 supernatant and cartilage (c) and horse 4 supernatant and cartilage (d)

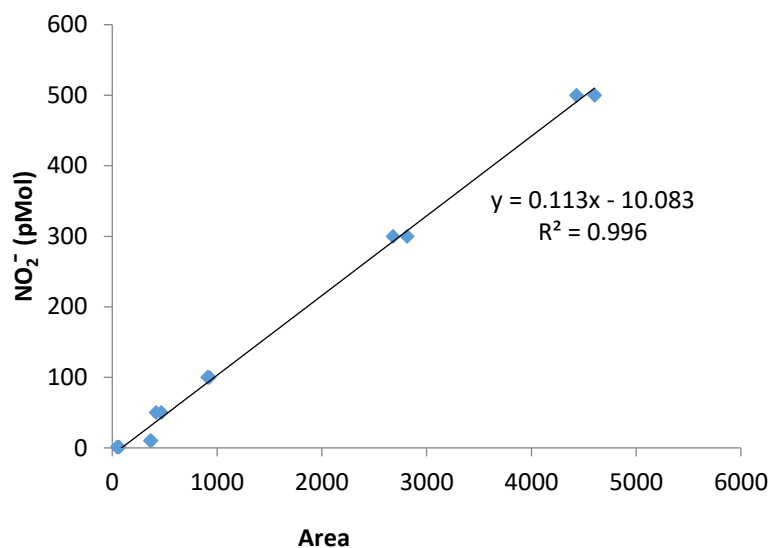


Figure 3: Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horses 1 to 3.

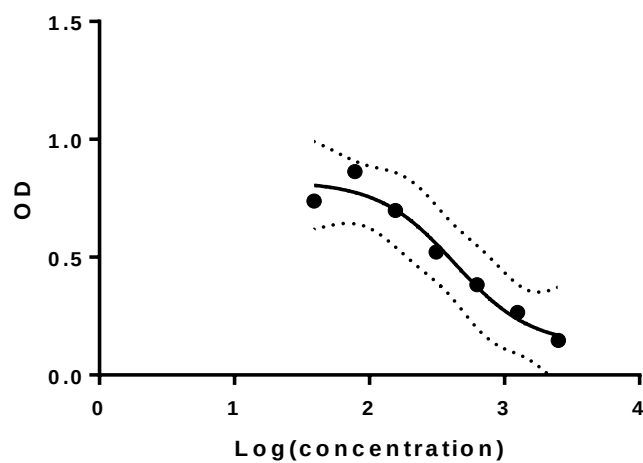


Figure 4: Four parameter logistic calibration curve fit for PGE_2 estimation in horses 1, 2 and 3.

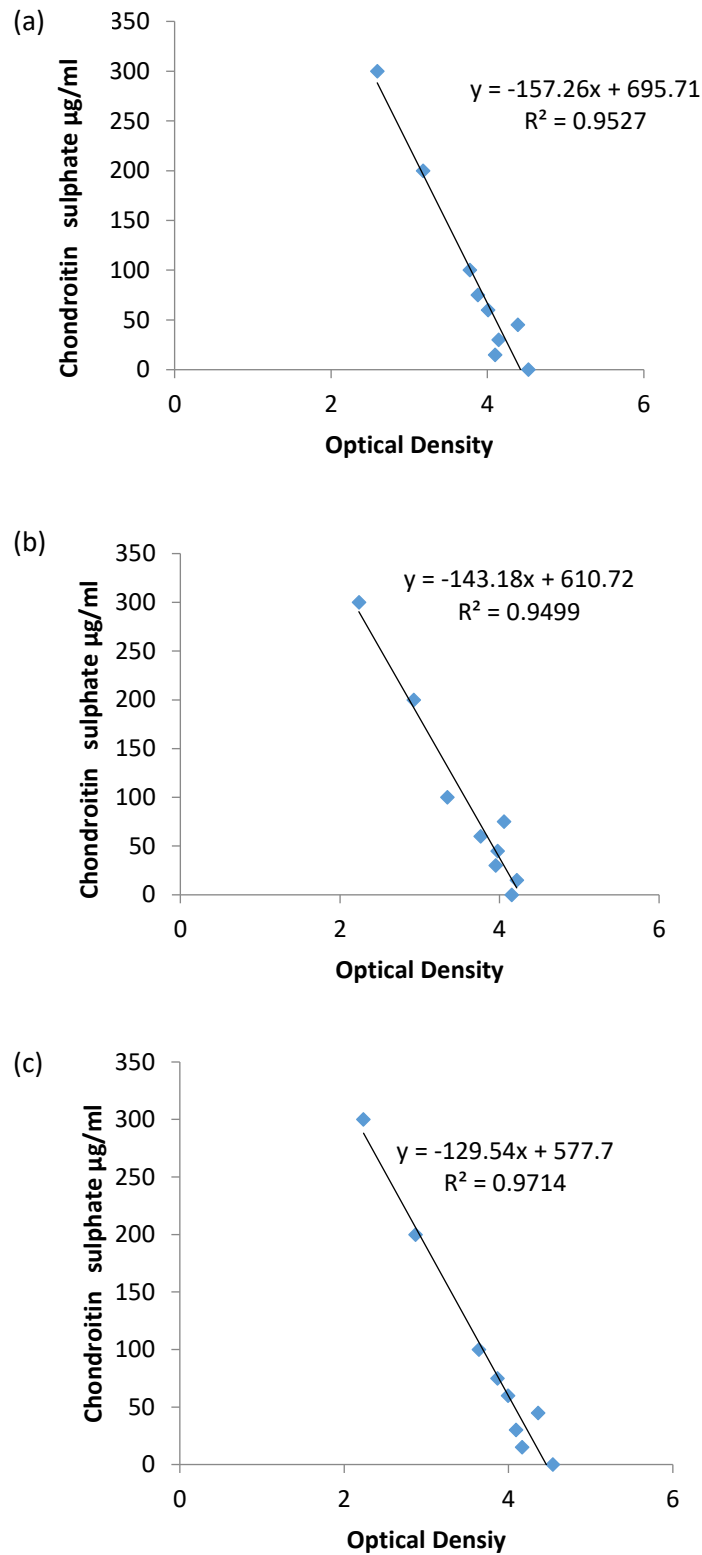


Figure 5 (a-c): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for in horse 1 supernatant and cartilage (a), horse 2 supernatant and cartilage (b) and horse 3 supernatant and cartilage (c).

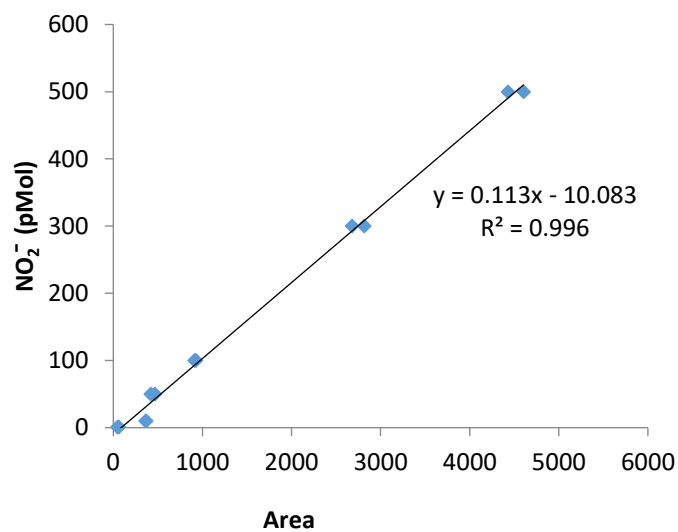


Figure 6: Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horses 1 to 3.

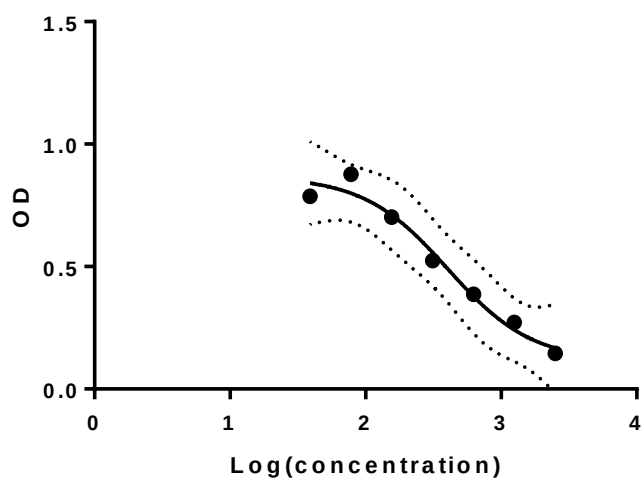


Figure 7: Four parameter logistic calibration curve fit for PGE₂ estimation in horses 1 to 3.

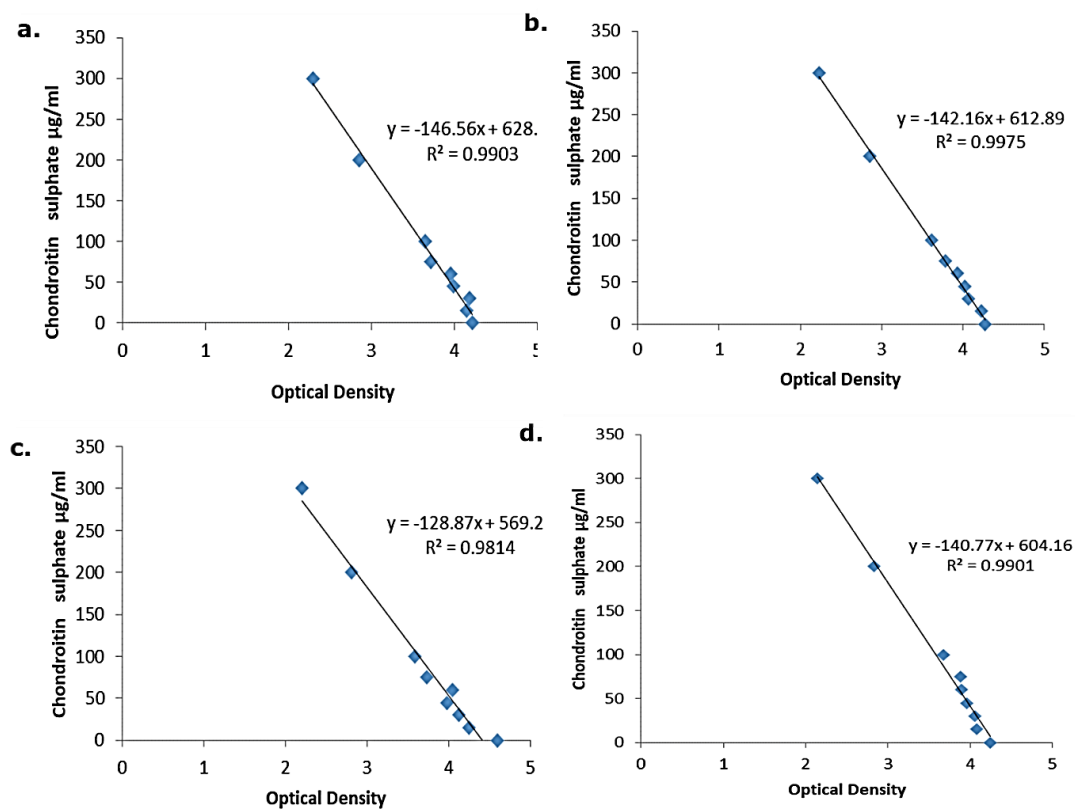


Figure 8 (a-d): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and horses 1 to 3 cartilage (d)

10.3 Appendix 3

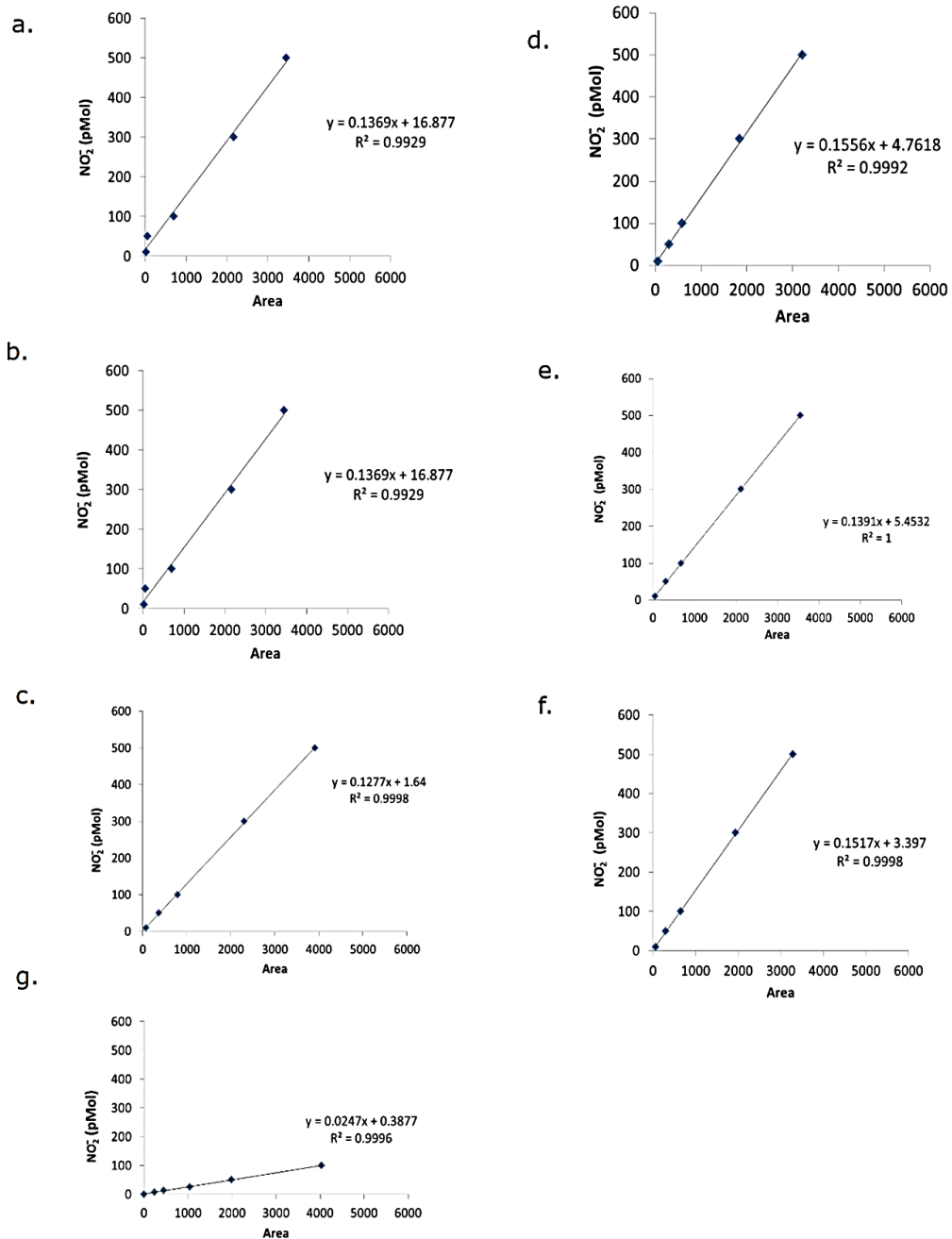


Figure 1: Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horse 1 (a), horse 2 (b), horse 3 (c), horse 4 (d), horse 5 (e), horse 6 (f) and horses 7 to 9 (g).

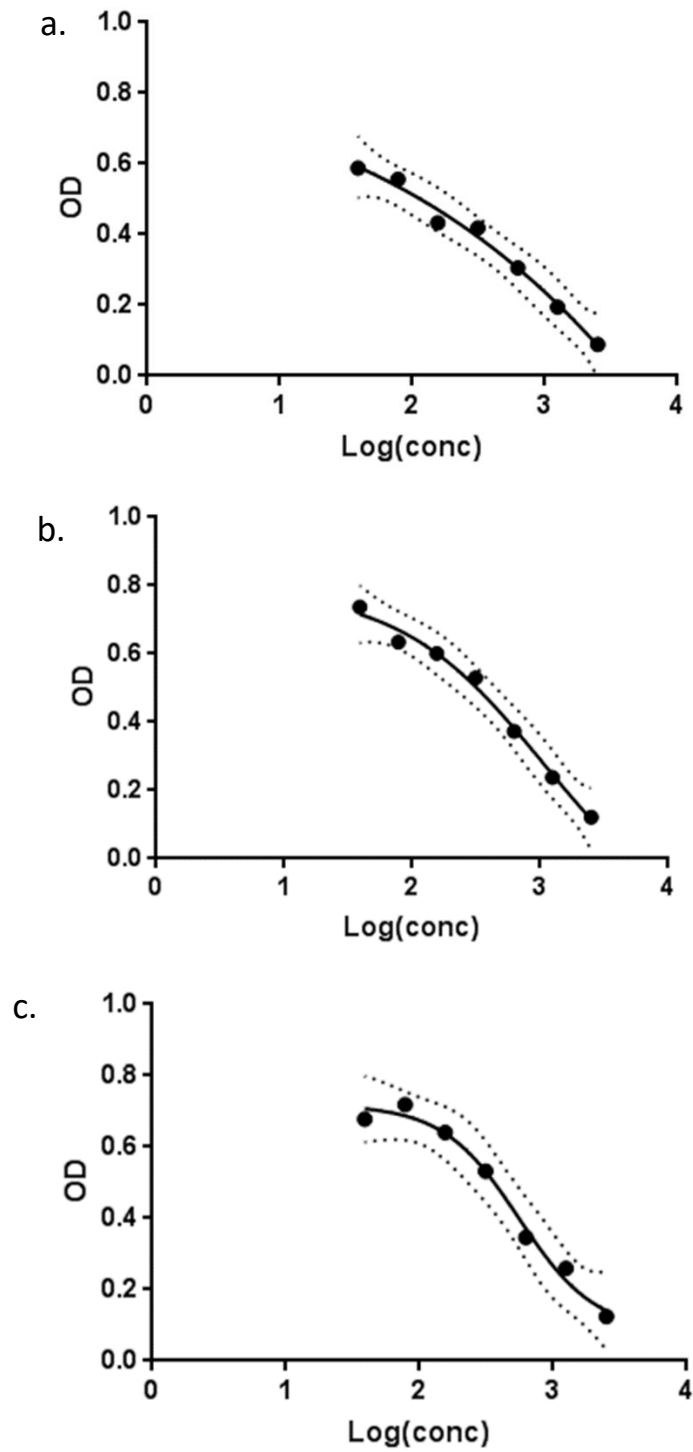


Figure 2: Four parameter logistic calibration curve fit for PGE₂ estimation in horses 1 to 3 (a), horses 4 to 6 (b) and horse 7 to 9 (c).

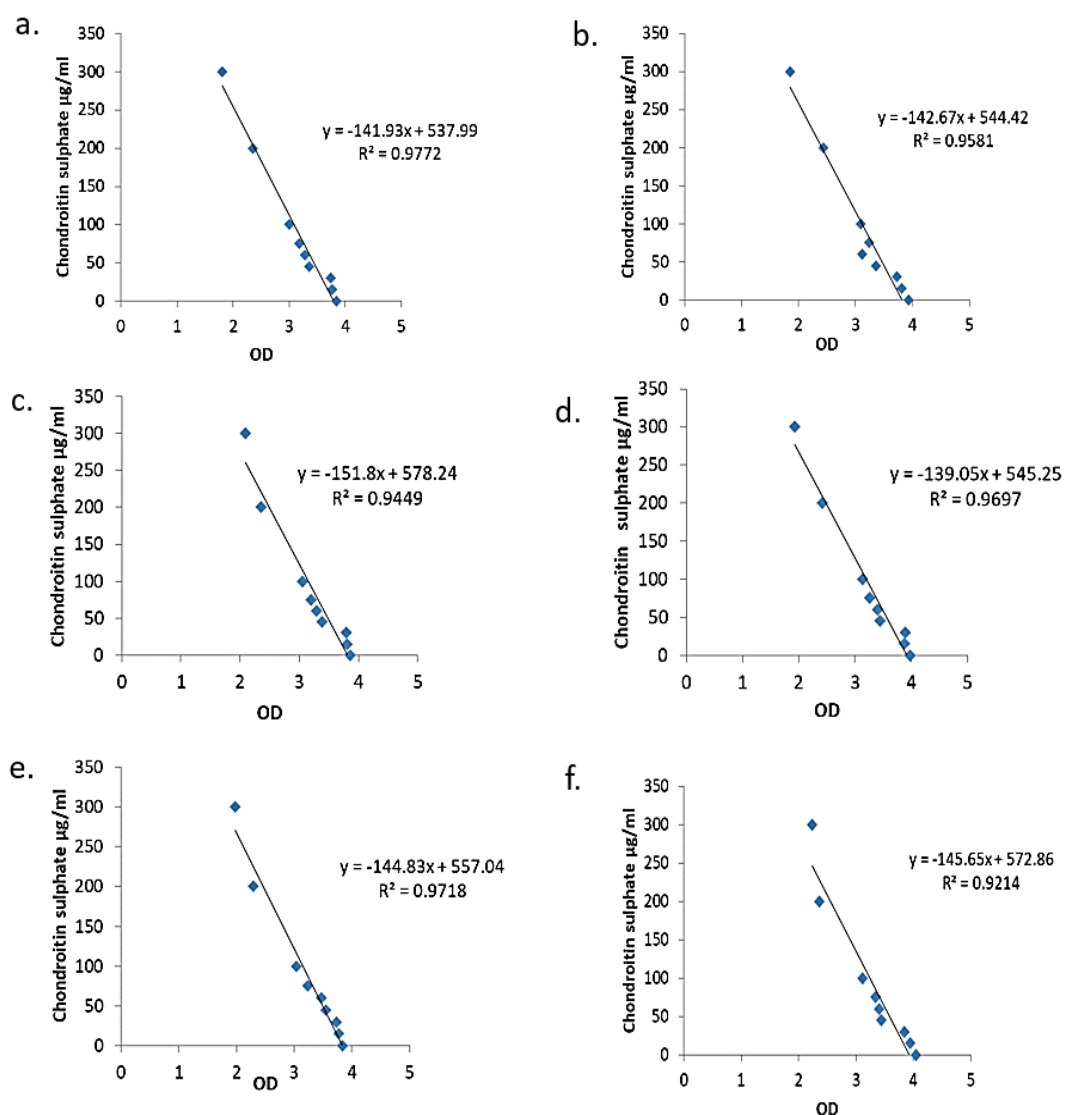


Figure 3 (a-f): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and cartilage (d) and horse 3 supernatant (e) and cartilage (f).

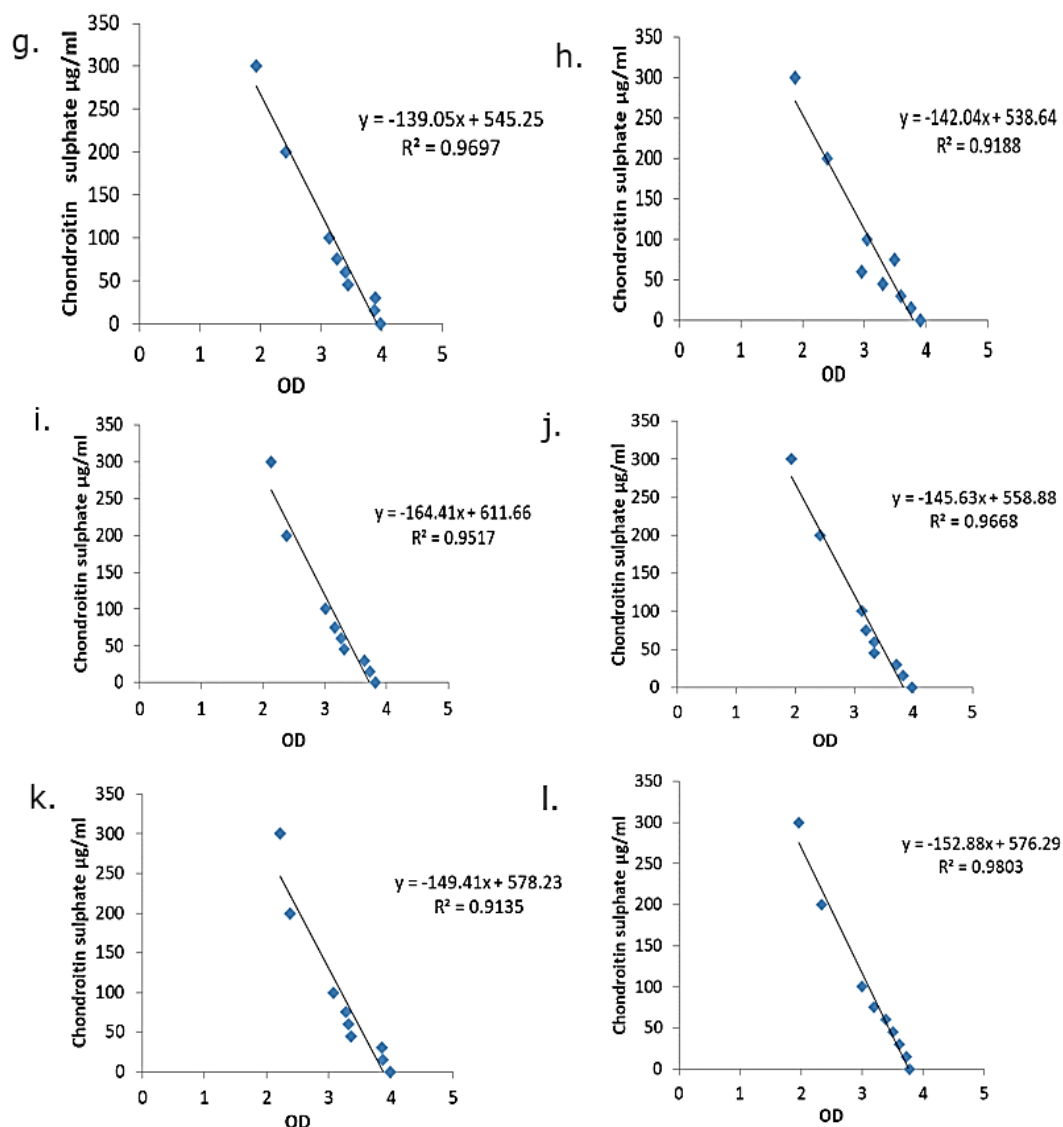


Figure 3 (g-l): Glycosaminoglycan (GAG) calibration curve. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 4 supernatant (g) and cartilage (h), horse 5 supernatant (i) and cartilage (j) and horse 6 supernatant (k) and cartilage (l).

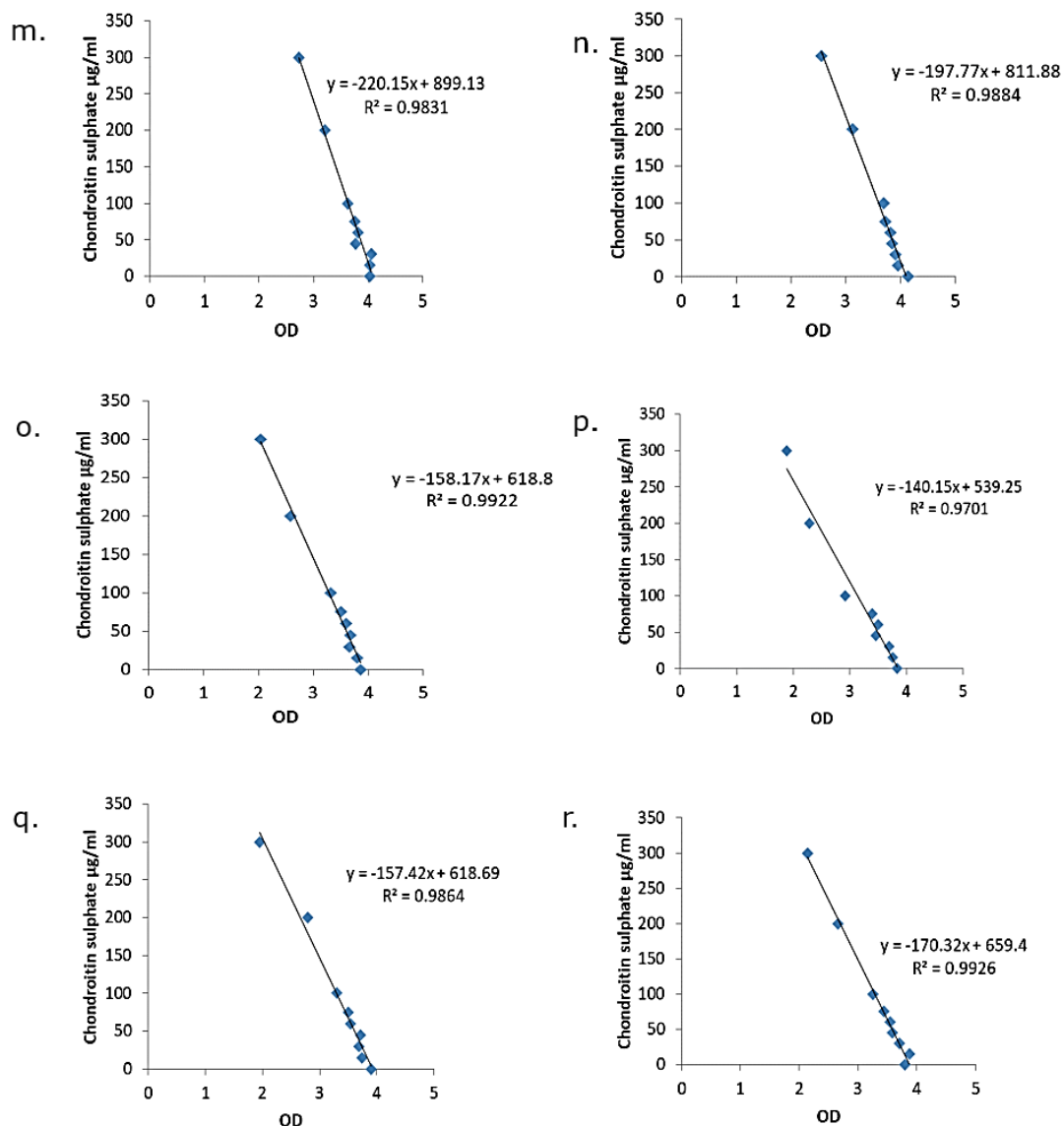


Figure 3 (m-r): Glycosaminoglycan (GAG) calibration curve. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 4 supernatant (m) and cartilage (n), horse 5 supernatant (o) and cartilage (p) and horse 6 supernatant (q) and cartilage (r).

10.4 Appendix 4

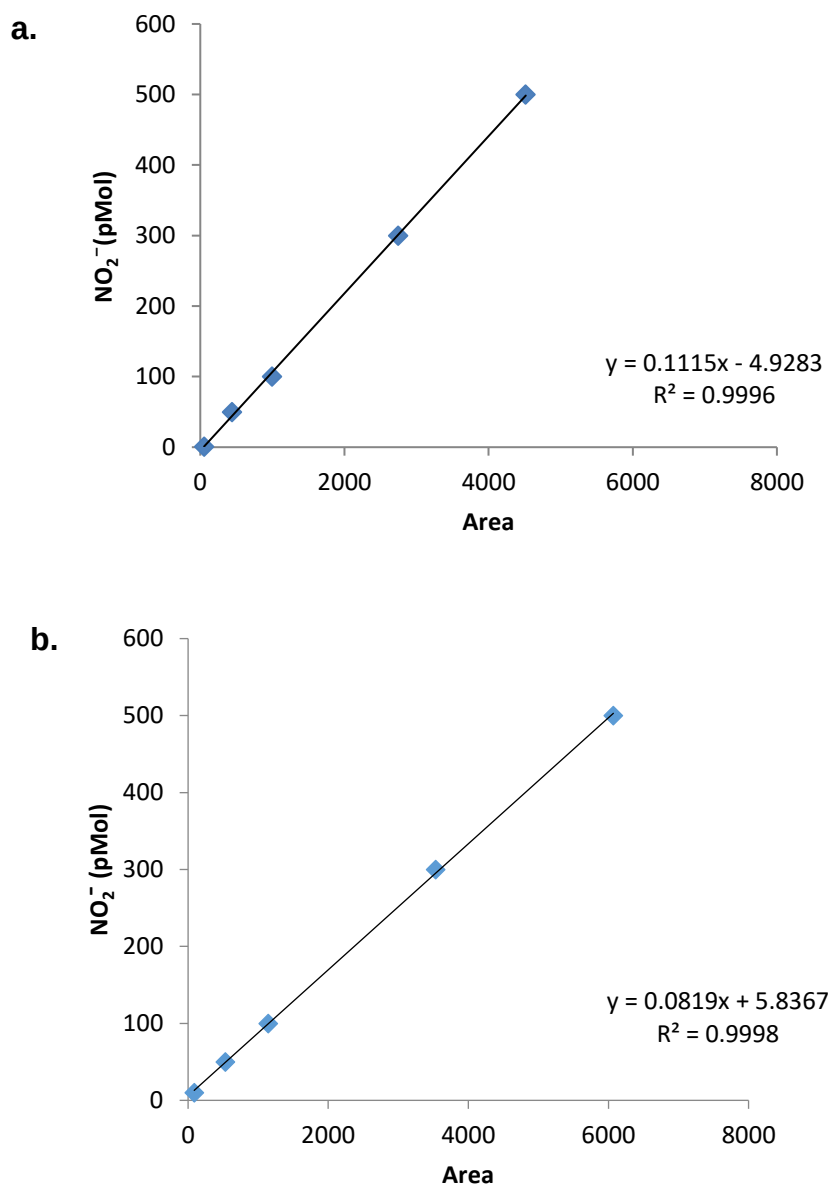


Figure 3 (a-b): Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horse 1, 2 and 3 (a) and horse 4, 5 and 6 (b).

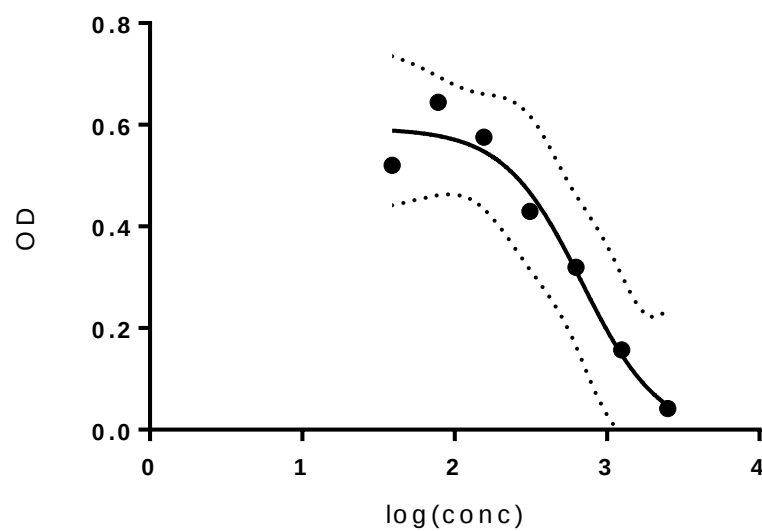


Figure 2: Four parameter logistic calibration curve fit for PGE₂ estimation in horses 1, 2 and 3.

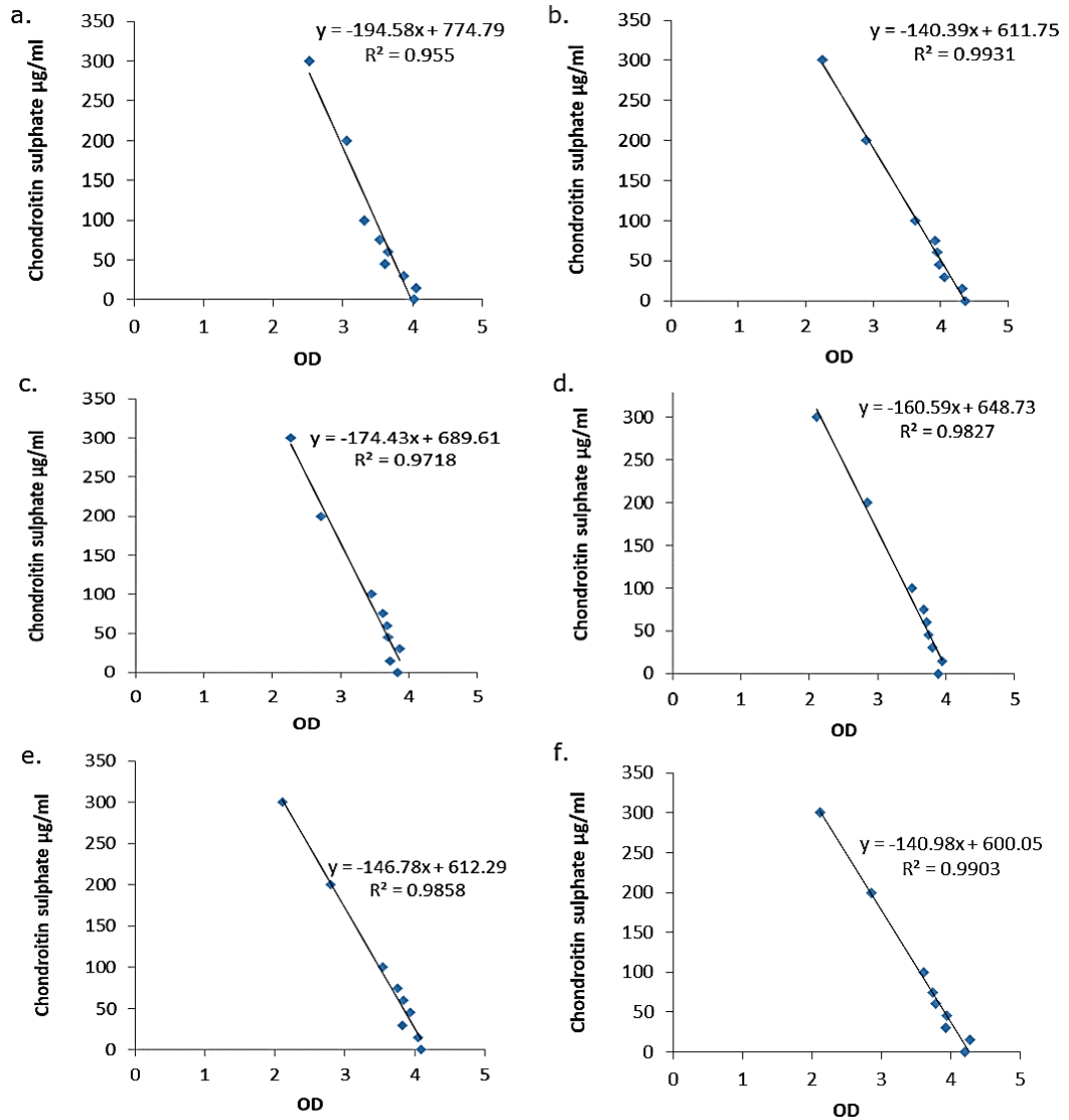


Figure 3(a-f): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and cartilage (d) and horse 3 supernatant (e) and cartilage (f).

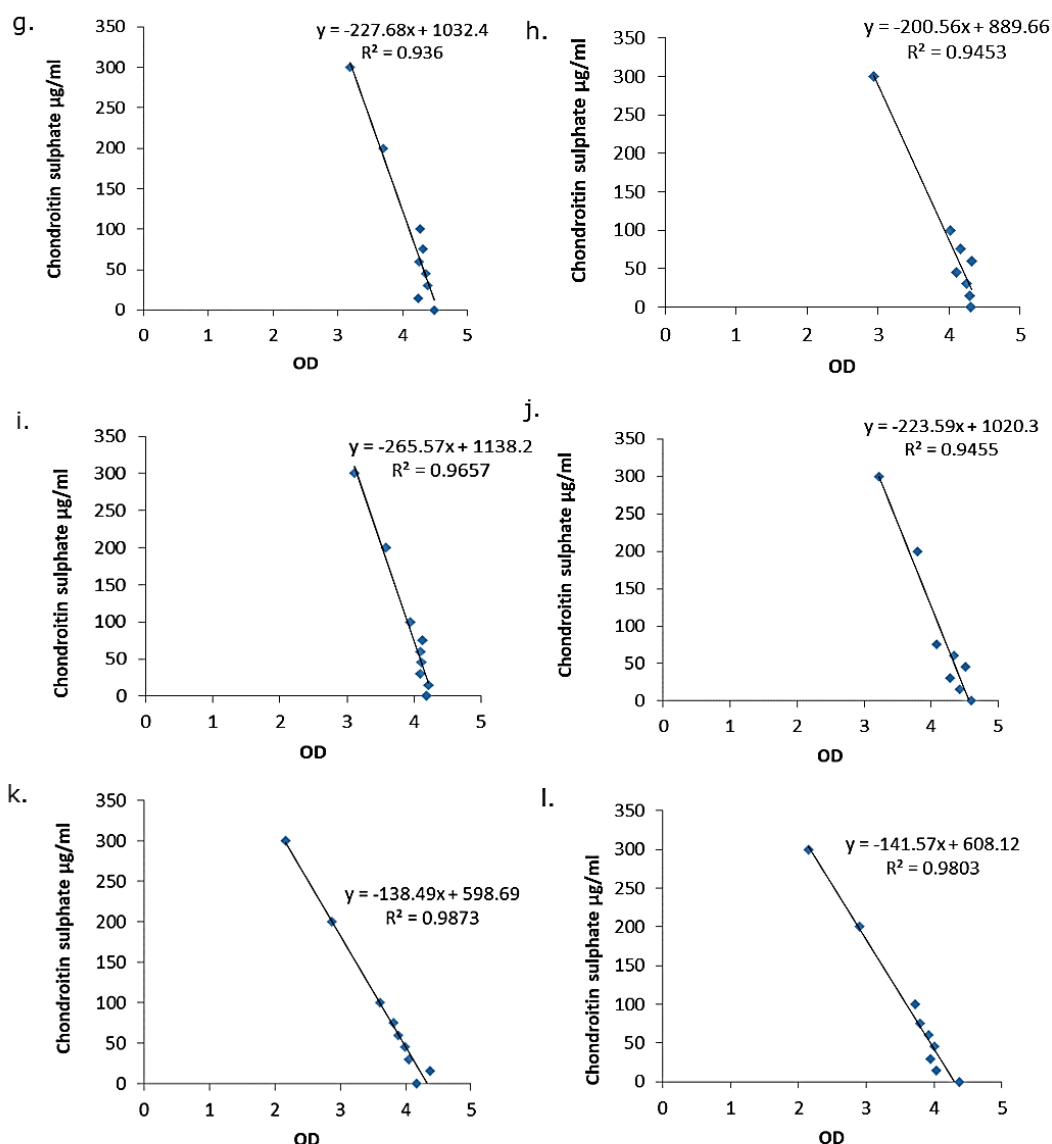


Figure 3 (g-l): Glycosaminoglycan (GAG) calibration curve. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 4 supernatant (g) and cartilage (h), horse 5 supernatant (i) and cartilage (j) and horse 6 supernatant (k) and cartilage (l).

10.5 Appendix 5

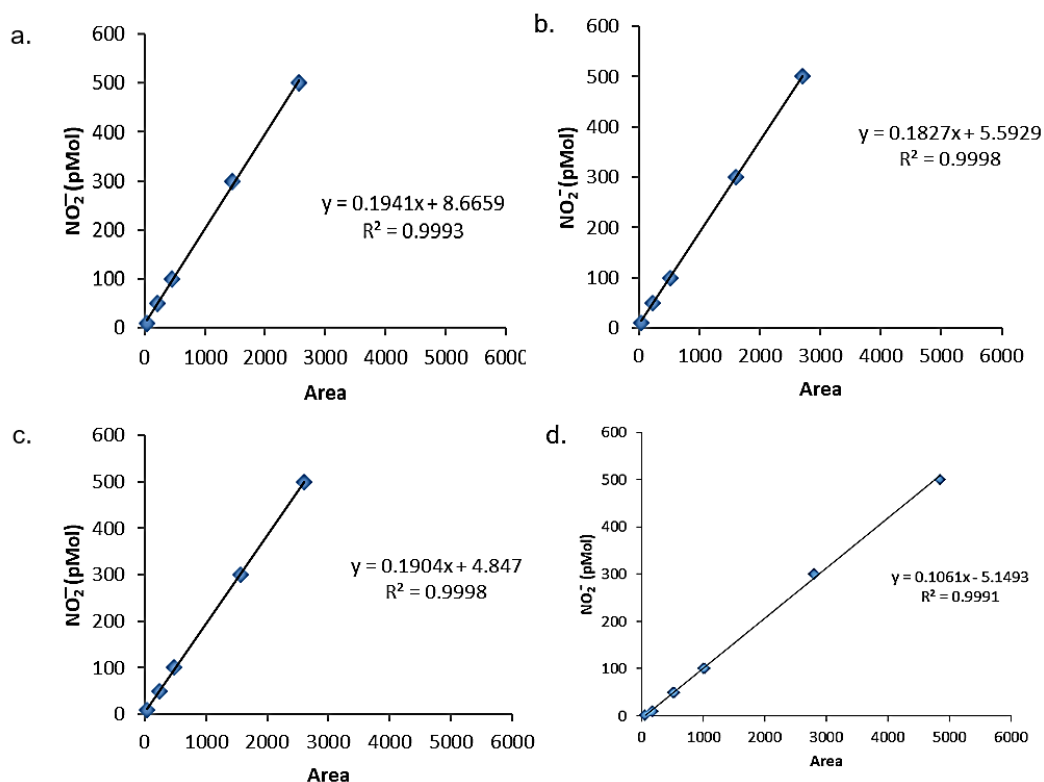


Figure 1 (a-d): Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horse 1 (a), horse 2 (b), horse 3 (c) and horses 4 to 6 (d).

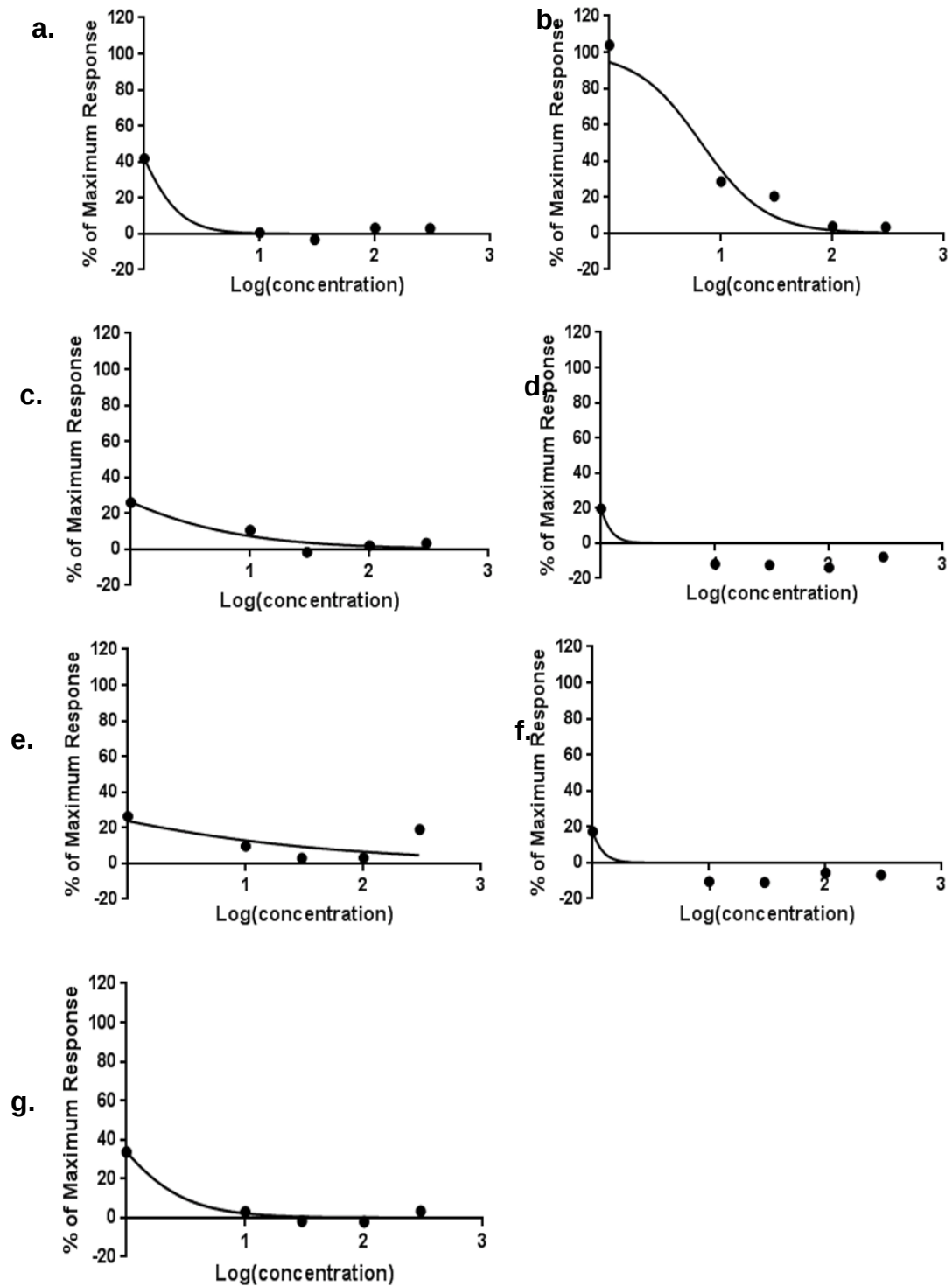


Figure 2: Dose-Response curve for all horses for suppression of IL-1 β stimulated nitrite release (a) horse 1, (b) horse 2, (c) horse 3, (d) horse 4, (e) horse 5, (f) horse 6, (g) average of all (n=6).

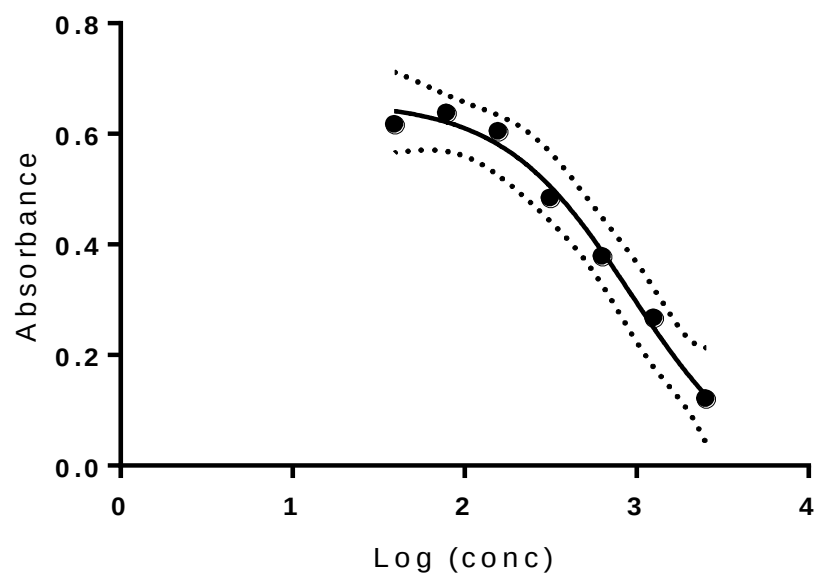


Figure 3: Four parameter logistic calibration curve fit for PGE₂ estimation in horses 1, 2 and 3.

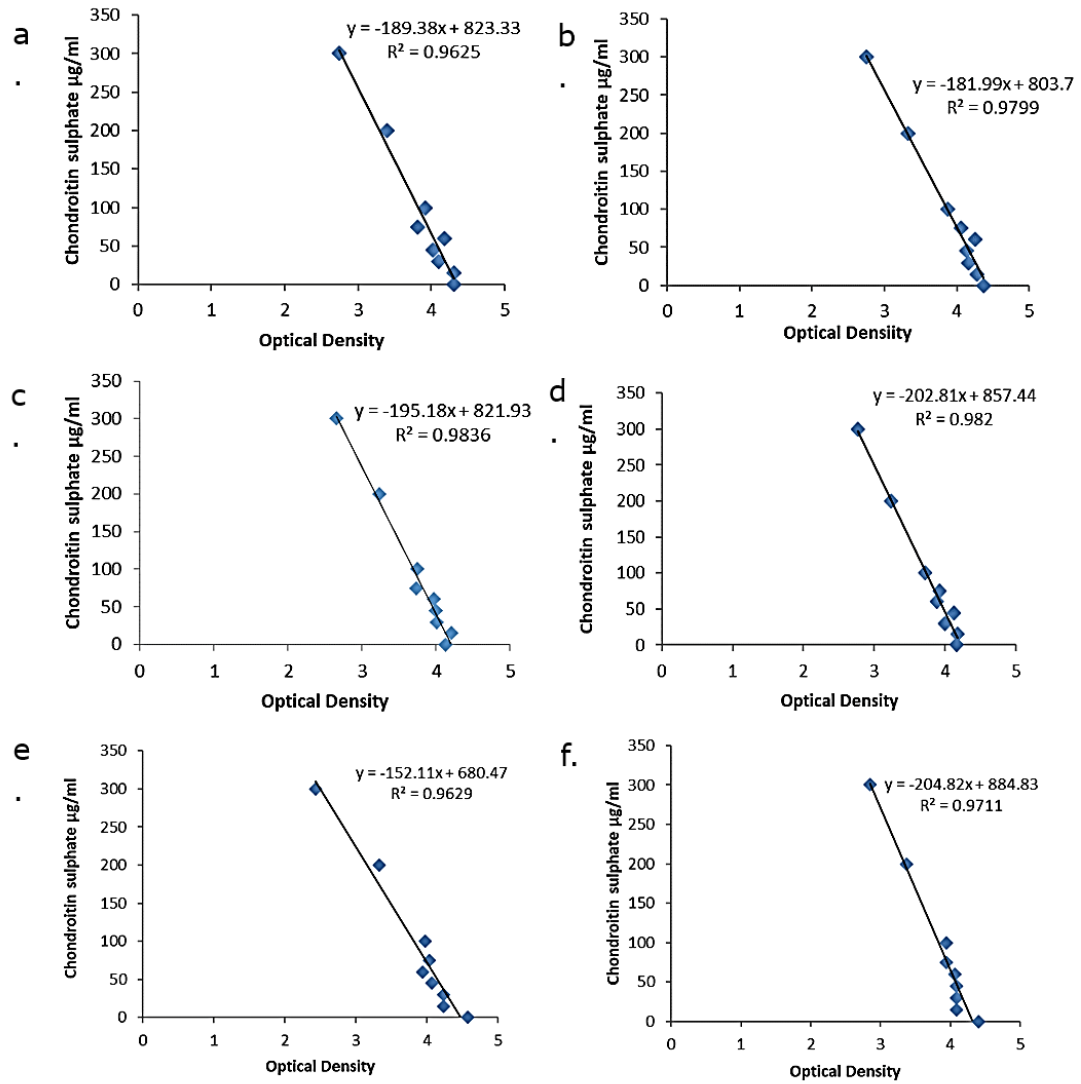


Figure 4 (a-f): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate (µg/ml). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and cartilage (d) and horse 3 supernatant (e) and cartilage (f).

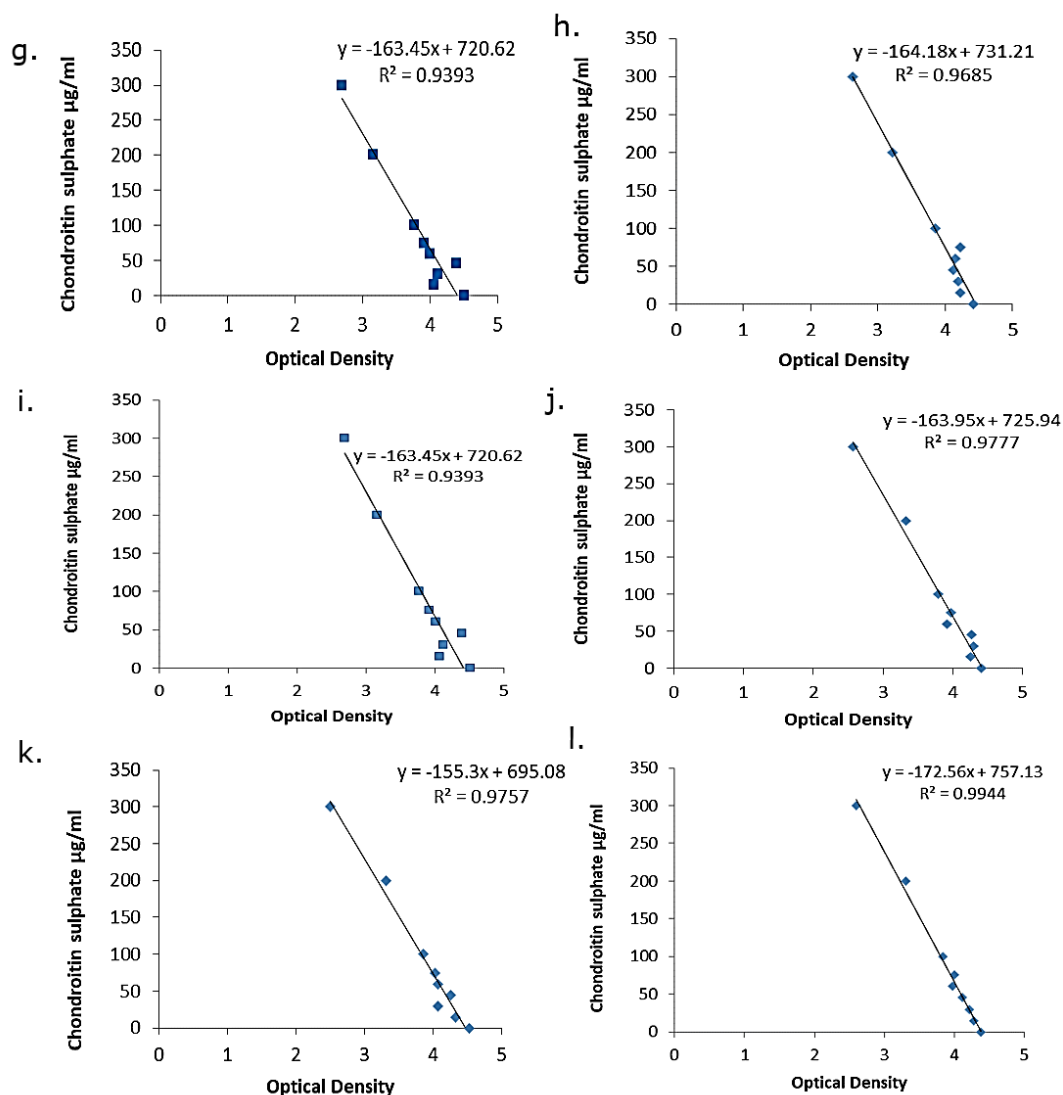


Figure 4 (g-l): Glycosaminoglycan (GAG) calibration curve. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 4 supernatant (g) and cartilage (h), horse 5 supernatant (i) and cartilage (j) and horse 6 supernatant (k) and cartilage (l).

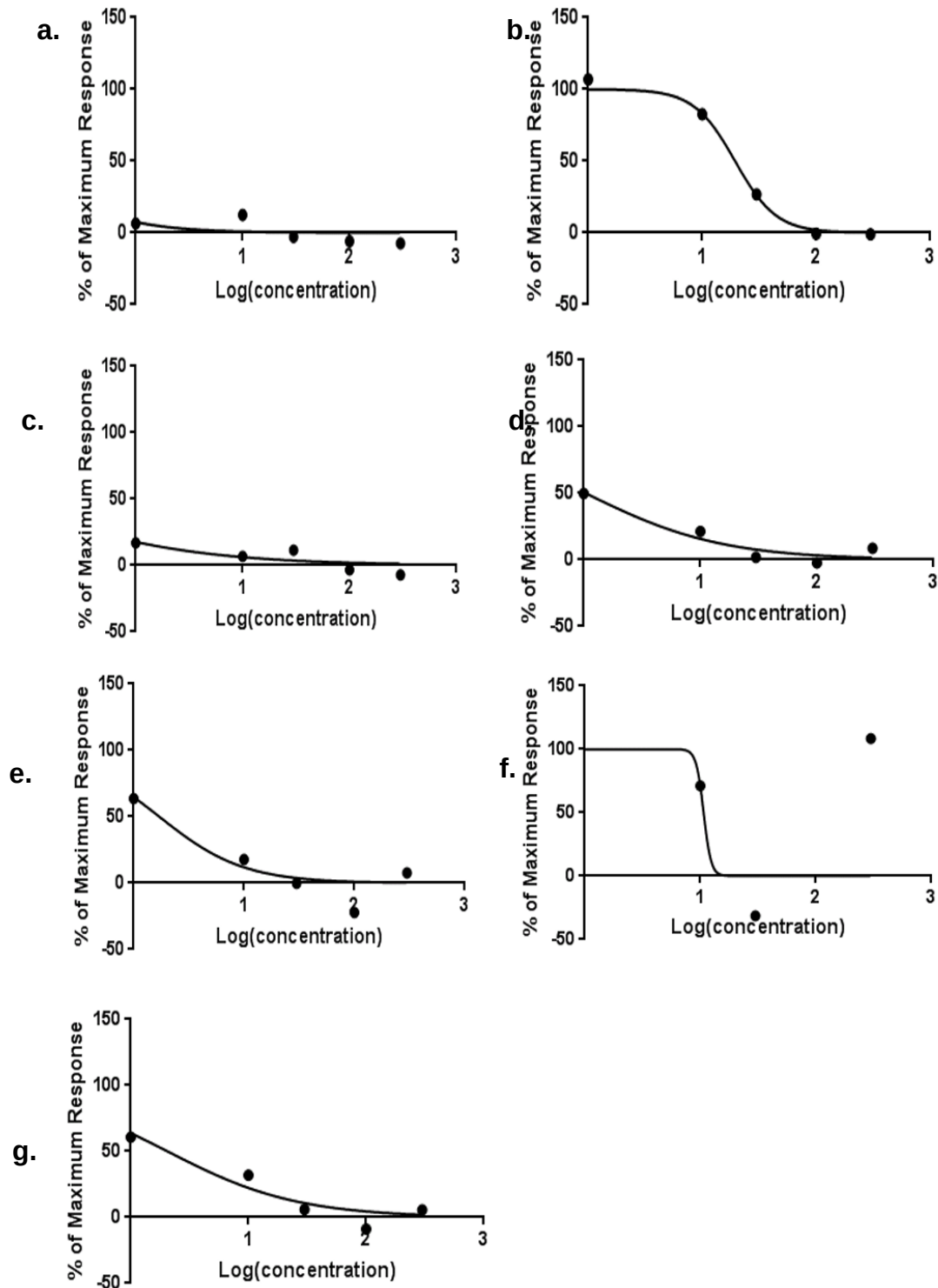


Figure 5: Dose response curve for all horses for suppression of IL-1 β stimulated GAG release (a) horse 1, (b) horse 2, (c) horse 3, (d) horse 4, (e) horse 5, (f) horse 6, (g) average of all (n=6).

10.6 Appendix 6

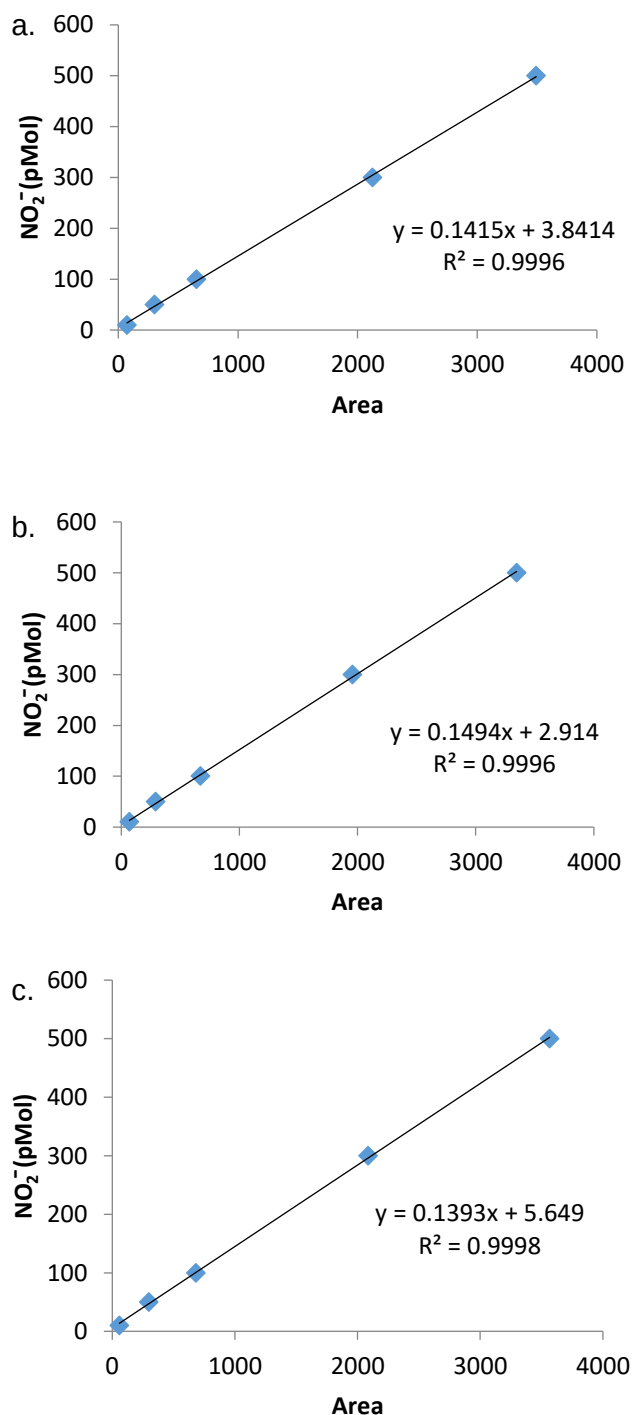


Figure 1 (a-c): Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horse 1 (a), horse 2 (b) and horse 3 (c).

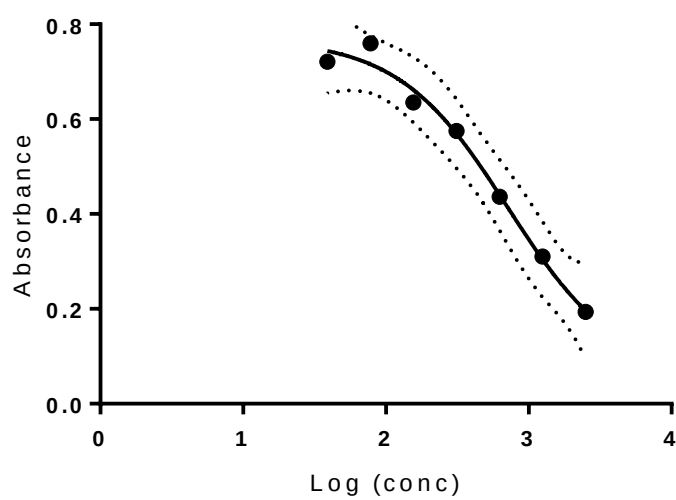


Figure 2: Four parameter logistic calibration curve fit for PGE₂ estimation in horses 1, 2 and 3.

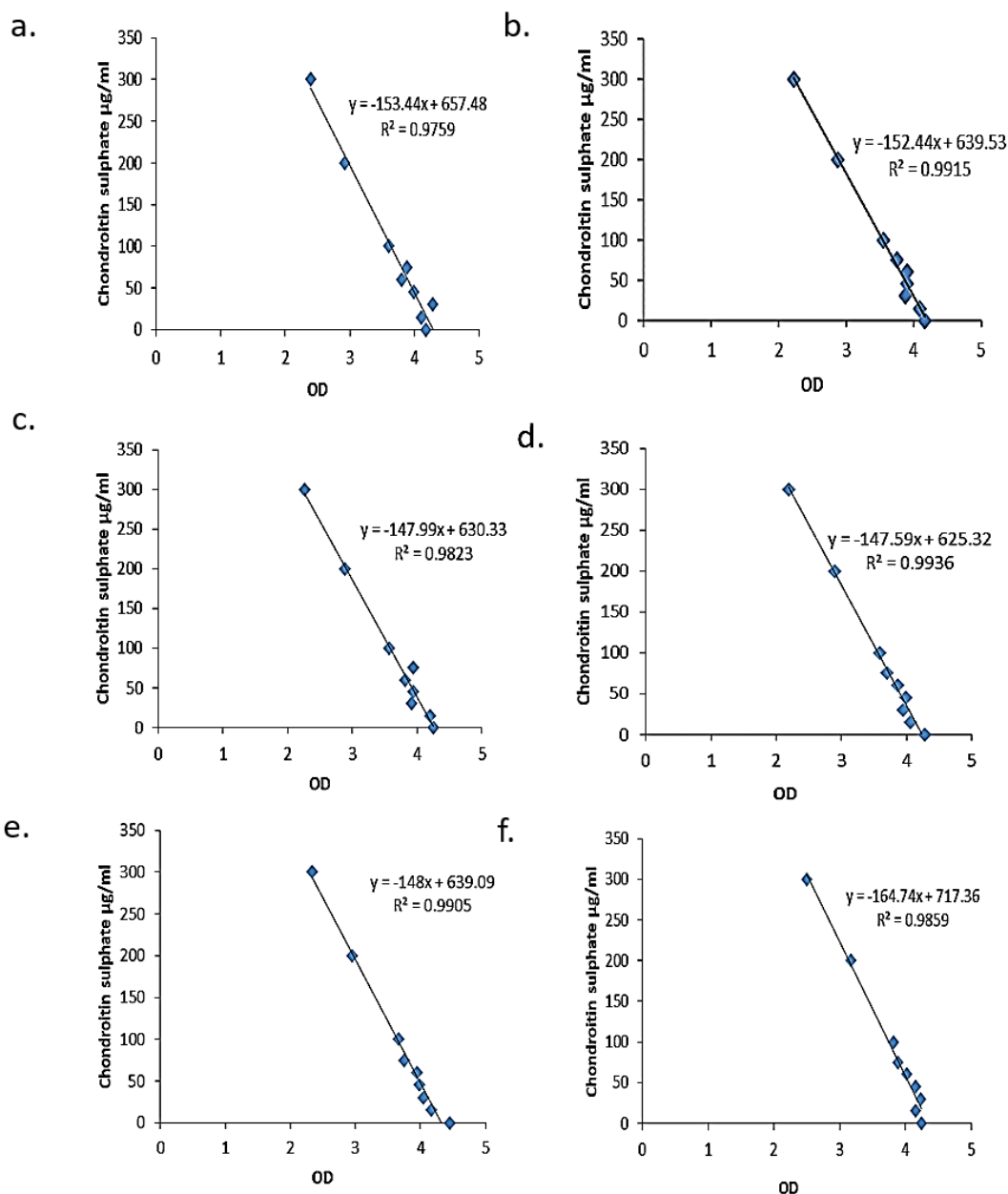


Figure 3 (a-f): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and cartilage (d) and horse 3 supernatant (e) and cartilage (f).

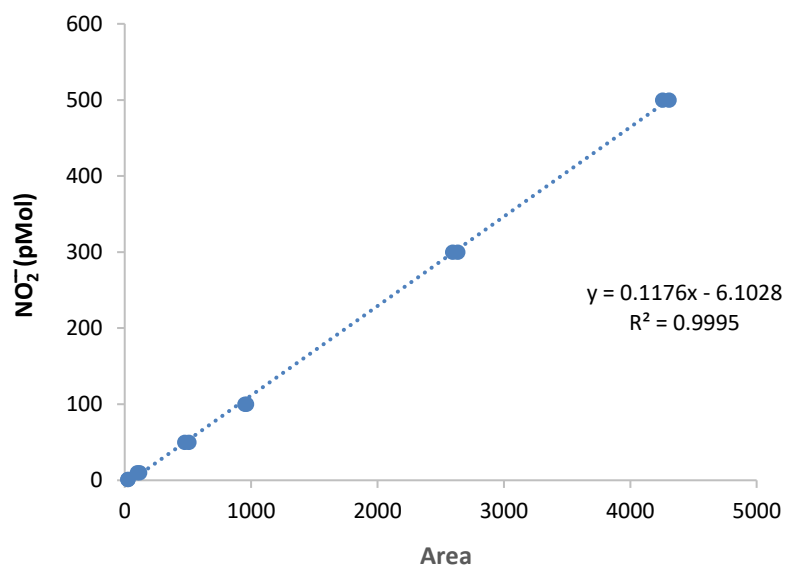


Figure 4: Nitrite calibration curve. X axis represents area and Y axis represents amount of nitrite used for standard curve in horses 1 to 3.

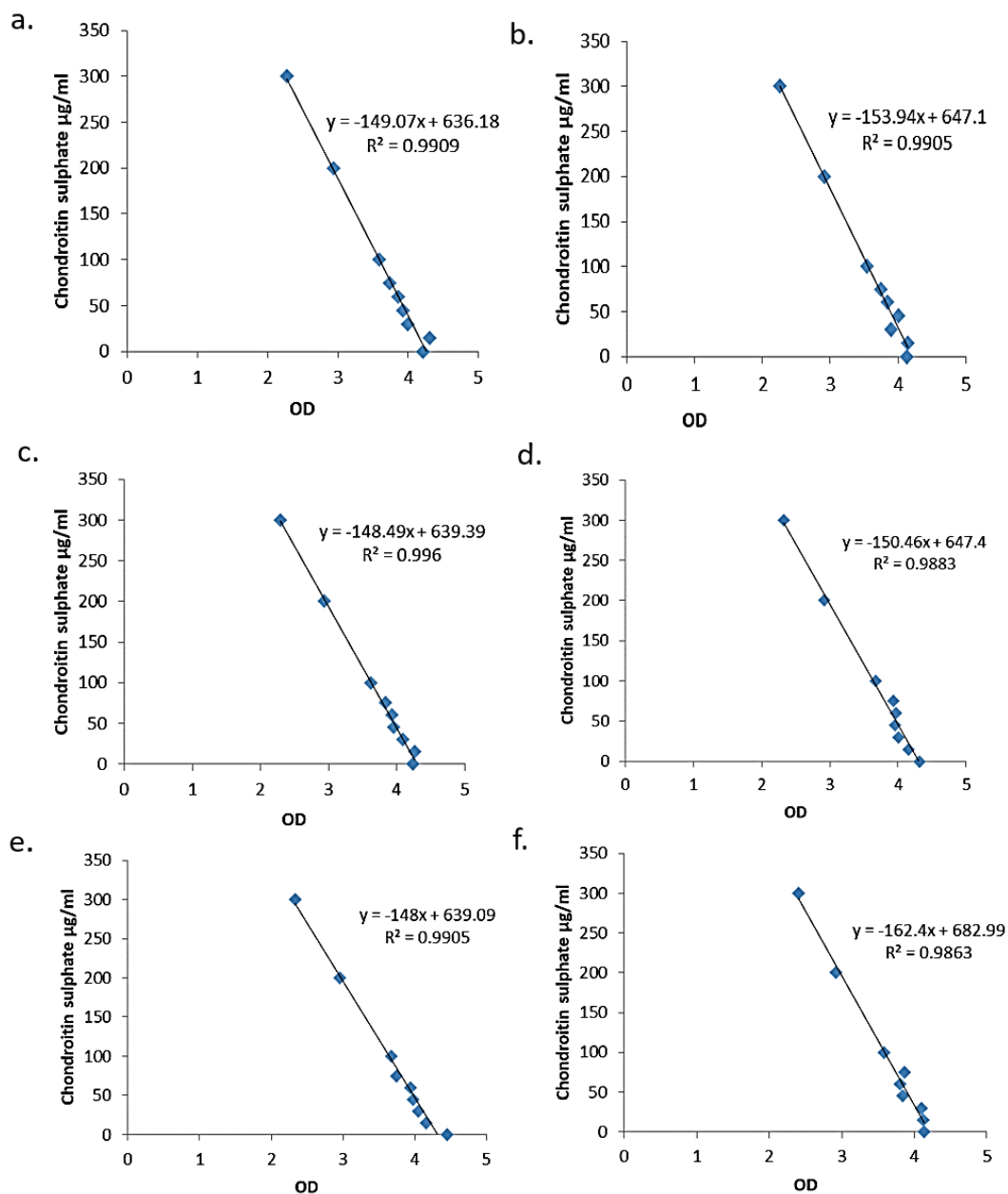


Figure 5 (a-f): Glycosaminoglycan (GAG) calibration curves. X axis represents optical density (OD) response and Y axis represents concentration of chondroitin sulphate ($\mu\text{g/ml}$). Used for horse 1 supernatant (a) and cartilage (b), horse 2 supernatant (c) and cartilage (d) and horse 3 supernatant (e) and cartilage (f).