An *in vitro* model to study cartilage metabolism in Kashin-Beck Disease

Thesis submitted in fulfilment of the requirements of the degree of Doctorate of Medicine, University of Cardiff

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SUMMARY OF THESIS: POSTGRADUATE RESEARCH DEGREES

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SECTION A: TO BE COMPLETED BY THE CANDIDATE AND SUBMITTED WITH THE THESIS

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Full Title of Thesis	**An *in vitro* model to study cartilage metabolism in Kashin-Beck Disease**

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Summary of Thesis:

Kashin-Beck disease is endemic chronic degenerative osteoarthropathy whose main pathological changes occur in the growth plate and articular cartilage of human limbs and joints, where it is manifest as cartilage degeneration and necrosis. The effects of the disease on the growth plate therefore alter growth in children and can lead to growth arrest (short stature and limbs) and developmental deformities (crooked joints). In adulthood patients present with pain and deformity commonly affecting the hand, wrist, elbow, knee and ankle with radiological features and joint pattern involvement similar to those seen in rheumatoid arthritis. Past and current research suggests that Kashin-Beck disease, with its endemic geographical distribution in China and Tibet, is due to the combined presence of fungal mycotoxins (found on the stored food ingested by affected populations) and a regional selenium deficiency in the environment providing local food sources. This evidence is supported by the correlation between the geographical occurrence of these 2 factors and the incidence of Kashin-Beck disease.

The objective of this study was to develop and *in vitro* cartilage culture system to mimic the changes seen in Kashin-Beck disease and thus determine the effects different sources of selenium, in the presence or absence of Nivalenol, had on cartilage neograft metabolism. Our hypothesis was that growth and metabolism of cartilage will be affected by exposure to either selenium or the mycotoxin Nivalenol or both in combination and these effects will mimic those found in Kashin-Beck disease.

Collectively, the results of this study suggest that Nivalenol is the major contributor to cartilage pathology in this *in vitro* system that mimics Kashin-Beck disease, and that these deleterious effects are largely independent from selenium supplementation.

CONTENTS

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Lastly, I wish to thank my wife Helen for all her support, especially the tutorial on how to use EndNote!

Abbreviations

BUT	Butenolide
CILP	Cartilage intermediate layer protein
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulphate
Da	Dalton
DMEM	Dulbecco's Modified Eagles Medium
DMMB	Dimethylmethylene Blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DS	Dermatan sulphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Fulvic acid
FACIT	Fibril associated collagens with interrupted triple helices
FBS	Foetal Bovine Serum
GPx	Glutathione peroxidise
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HA	Hyaluronan
HCl	Hydrogen chloride
HPR	Hydroxyproline
HS	Heparan sulphate

IGD	Intergloblar domain
IL	Interleukin
KBD	Kashin-Beck Disease
kDa	Kilo Dalton
KS	Keratan sulphate
mAb	Monoclonal antibody
MMP	Matrix metalloproteinase
MON	Moniliformin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na-Sel	Sodium Selenite
NIV	Nivalenol
NO	Nitric oxide
OA	Osteoarthritis
PBS	Phosphate buffered saline
PG	Proteoglycan
PHG-px	Phospholipid hydroperoxide glutathione peroxidise
PMSF	Phenyl methyl sulfonyl fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Se or SEL	Selenium
Sel-Met	Selenium-l-Methionine
Se-p	Selenoprotein P
sGAG	Sulphated Glycosaminoglycan
SLRPs	Small leucine-rich proteoglycans
TGF-β	Transforming Growth Factor β
TNF-α	Tumour necrosis factor α

CHAPTER ONE
INTRODUCTION

1.1 Composition of Normal Articular Cartilage

Articular cartilage is important in biomechanics and transmits weight-bearing forces between long bones and facilitates diarthrodial joint articulation. Articular cartilage is a specialised connective tissue with a large amount of extracellular matrix (ECM) and once skeletal maturation is reached composes of approximately 70% water, 20% collagen, 7% proteoglycans. The cartilage is resistant to wear and the hydrated, macromolecular mesh of the cartilage ECM aids in absorbing stress under mechanical load and provides a load-bearing surface to facilitate a low friction movement of the joint. Present within matured articular cartilage are chondrocytes which are embedded in the ECM of collagens and proteoglycans. These cells are responsible for the organisation of the ECM to provide a unique and highly organised cartilage tissue. Normally articular cartilage is an avascular, aneural tissue therefore making it more difficult for damage to occur.

1.2 Chondrocytes

Chondrocytes represent approximately 1% of the volume of articular cartilage (Vanwanseele, Lucchinetti et al. 2002). These cells are usually located within the lacunae and embedded within extensive ECM and have no cell-cell contact or interaction (Martin and Buckwalter 1998). Chondrocytes live for a long period of time and do not normally divide during adult life. Nutrients are obtained via the synovial fluid and they differ in size, shape and metabolic activity depending on which cartilage zone they originate from (Aydelotte, Mok et al. 1996). The role of chondrocytes is to maintain the integrity of the tissue through normal turnover of the ECM. To carry out this task they must be able to locate changes in the composition of the ECM either through degradation or demands placed on the joint surface, and evaluate the amount and types of ECM components to synthesise. The activity and function of chondrocytes differ during stages of skeletal development. During skeletal growth, the cells produce new cartilage to expand and remodel. However, once matured the

chondrocytes do not significantly alter the tissue volume but maintain the normal articular cartilage homeostasis (Buckwalter 1995)

1.3 Articular Cartilage Morphological Zones

Mature articular cartilage is a heterogeneous tissue that can be divided into 4 distinct zones; superficial, middle, deep and calcified zones (Figure 1.1). Each zone has physiological differences, varying in respect to water, proteoglycan and collagen concentrations and in aggregate size. The chondrocytes from each distinct zone also differ in size, shape, density and metabolic activity (Buckwalter 1999) However, it is important to note that during development these zones do not exist with the tissue there being unstratified with randomly distributed cells.

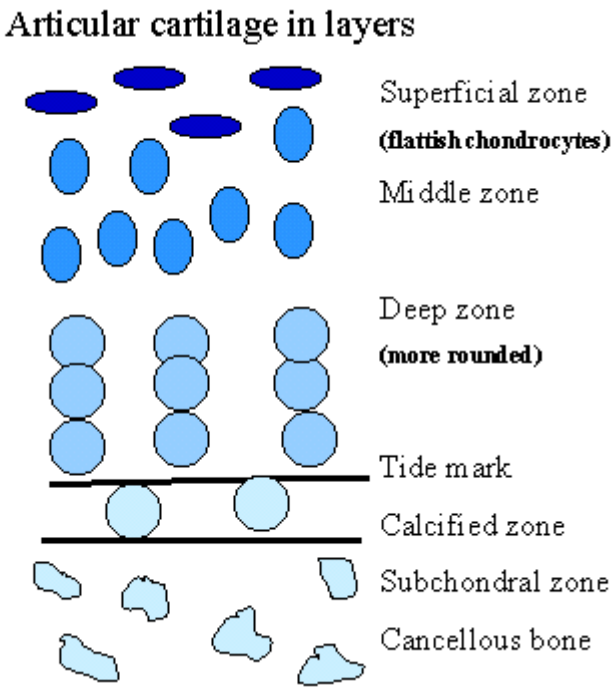


Figure 1.1 The distinct zones of articular cartilage

1.4 The Superficial Zone

The superficial zone (Figure 1.1), the thinnest of all four zones, is the nearest to the cartilage surface and comprises of an acellular and deeper cellular layer. The acellular layer at the surface, also known as the *lamina splendens* (Mac 1951) consists of a thin layer of amorphous material overlying a sheet of fine fibrils, with suggestions of areas containing type I collagen (Duance 1983). The lower cellular region contains chondrocytes that are flat or ellipsoid in shape which lie parallel to the surface of the cartilage. The ECM of the superficial zone contains low amounts of proteoglycan but is rich in collagen. These collagen fibrils lie parallel to the cartilage surface, providing high strength and tensile stiffness. There is also a high proportion of water and fibronectin in this zone compared to the other zones (Buckwalter 1999).

1.5 The Transitional Zone

The transitional zone (Figure 1.1) is the middle zone that lies in between the superficial and deep zones. This zone makes up 40-60% of the articular cartilage content. Chondrocytes within the transitional zone are spherical in shape and more randomly organised. The cells in this zone contain a higher concentration of synthetic organelles and the synthesised ECM contain collagen fibrils that are larger in diameter and consists of higher amounts of proteoglycan, but less collagen and water (Buckwalter 1999).

1.6 The Radial or Deep Zone

Chondrocytes within this zone are aligned in columns of lacunae perpendicular to the surface of the cartilage. The collagen fibrils within this deep zone are the largest in diameter compared to the other cartilage zones but contain the lowest overall amount of collagen but highest quantity of proteoglycan. The collagen fibrils in this zone emerge from the underlying calcified layer and anchor themselves (Vanwanseele, Lucchinetti et al. 2002).

1.7 The Zone of Calcified Cartilage

The calcified cartilage zone is the borderline zone between the cartilage and subchondral bone. The chondrocytes within this layer are much smaller in size and volume and contain lesser amounts of organelles. These cells also have a lower metabolic activity since they are often surrounded by calcified cartilage, and are thought to have the potential to contribute to the progression of joint diseases such as osteoarthritis (Walker, Fischer et al. 1995). This zone contains the "tidemark"; a basophilic line which straddles the boundary between calcified and uncalcified cartilage.

1.8 Extracellular Matrix

Collagen fibrils and proteoglycans are the structural components of the articular cartilage ECM, supporting the internal mechanical stresses that result from loads applied to the articular surface. There are also smaller amounts of other non-collagenous proteins present within ECM (discussed later). The ECM of articular cartilage is arranged into regions around the chondrocytes; pericellular (closest to the cells), territorial (extending around single or groups of cells) and the interterritorial matrix (furthest away from the cells). The chondrocytes are responsible for the synthesis of the ECM components.

1.9 Biochemistry of Proteoglycans

Proteoglycans represent a large family of poly-anionic glycoconjugates. These molecules are deemed to play metabolic, functional and structural roles in both soft and mineralised tissues of the body and are present in a wide variety of non-vertebrate and vertebrate species. Proteoglycans can be characterised as having a central core protein to which one or more long glycosaminoglycan (GAG) chains, with repeating disaccharide sub-units, covalently attach through a specific sequence of linkage region trisaccharides (Roden and Armand 1966). The physical and biological characteristic functions of proteoglycans are partly determined by the characteristics of the GAG chain and partly by the structure of the core protein involved in specific interactions with other ECM molecules.

1.10 Glycosaminoglycans (GAG)

Proteoglycans contain one or more types of GAG covalently linked to the same core protein (Heinegard 1977). The GAG component of proteoglycans can be defined as a highly anionic, linear polysaccharide that consists of repeating disaccharide units of hexosamine (either D-glucosamine or D-galactosamine) and a hexuronic acid (D-glucuronic acid or iduronic acid). The only exception is keratan sulphate, which is composed of hexosamine (glucosamine) and galactose. Characterisation of the disaccharide sequence has defined six different types of GAG in vertebrate tissues, namely dermatan sulphate (DS), chondroitin sulphate (CS), keratan sulphate (KS), heparin and heparan sulphate (Hep/HS) and hyaluronan (HA) (Table 1.1 and Figure 1.2).

GAGs are generally secreted into the ECM covalently bound to core proteins and they are variously sulphated. One exception is HA which is not covalently attached to core proteins and is non-sulphated (Prehm 1984). The attachment of the corresponding core protein occurs through a linkage region. CS, DS, HS and heparin all attach to the specific protein core through a reducing terminal of the GAG polysaccharide and the hydroxyl group of serine residues in the protein core via an O-glycosidic bond (Muir 1958). A tetra-saccharide linkage region sequence of gluconic acid-galactose-galactose-xylose comprises the linkage region and the xylose covalently binds to the serine residue on the protein core (Forsee and Roden 1981). Additionally, those serine residues carrying GAG chains are thought to be invariably located next to glycine residues in the protein, as serine-glycine-X-glycine sequences. These sequences have also been proposed to be prerequisites for the attachment of the GAG to the protein core (Bourdon, Krusius et al. 1987). KS, however, does not share this form of linkage and it is either O-linked to serine via N-acetylglucosamine or N-linked to asparagine residues via N-acetylglucosamine (Nilsson, Nakazawa et al. 1983). GAGs contain sulphate groups and carboxyl groups, which render these macromolecules highly negative under physiological conditions (Bartold 1987). Thus, selective epimerisation of the D-glucuronic acid in DS, HS and heparin and the selective degree of sulphation within the GAGs allow for greater diversification, leading to the production of GAG chains with numerous specific properties. GAGs present within cartilage are no exception with differences in chain length and variation in the degree of sulphation with respect to quantity and position. For example, the CS GAG chain may be sulphated at the 4- or 6- position of

the galactosamine residues. The sulphated status is loosely dependent on tissue type, age and pathology.

1.11 Proteoglycan core protein

The number of GAGs covalently linked to a protein core varies from 1-150, and also vary in length from a couple to hundreds of disaccharide units (Kreis and Vale, 1993). Additionally, the molecular weight of the core protein can vary from 10kDa to 400kDa (Kreis and Vale, 1993). With the advances of biochemical and molecular techniques, it has been demonstrated that there are distinct differences between proteoglycan families. For this reason, proteoglycans of the ECM have been classified according to their core protein and relative biological function (Kreis and Vale, 1993); (Iozzo 1997).

1.12 Proteoglycans of Articular Cartilage

Hyaline cartilage contains several well-characterised proteoglycans; several of these are discussed below. The largest proteoglycan is aggrecan consisting over a 100 CS and 50 KS side chains. The other proteoglycans (e.g. small leucine-rich proteoglycans- SLRPs) present are much smaller in size and are able to interact with collagen fibrils within the ECM. These include decorin, biglycan and fibromodulin. In addition minor amounts of versican and perlecan are also present at different stages of cartilage development. Another molecule that can also be considered as a member of proteoglycan family is type IX collagen as its α2(IX) chain may bear a CS GAG chain (Roughley and Lee 1994).

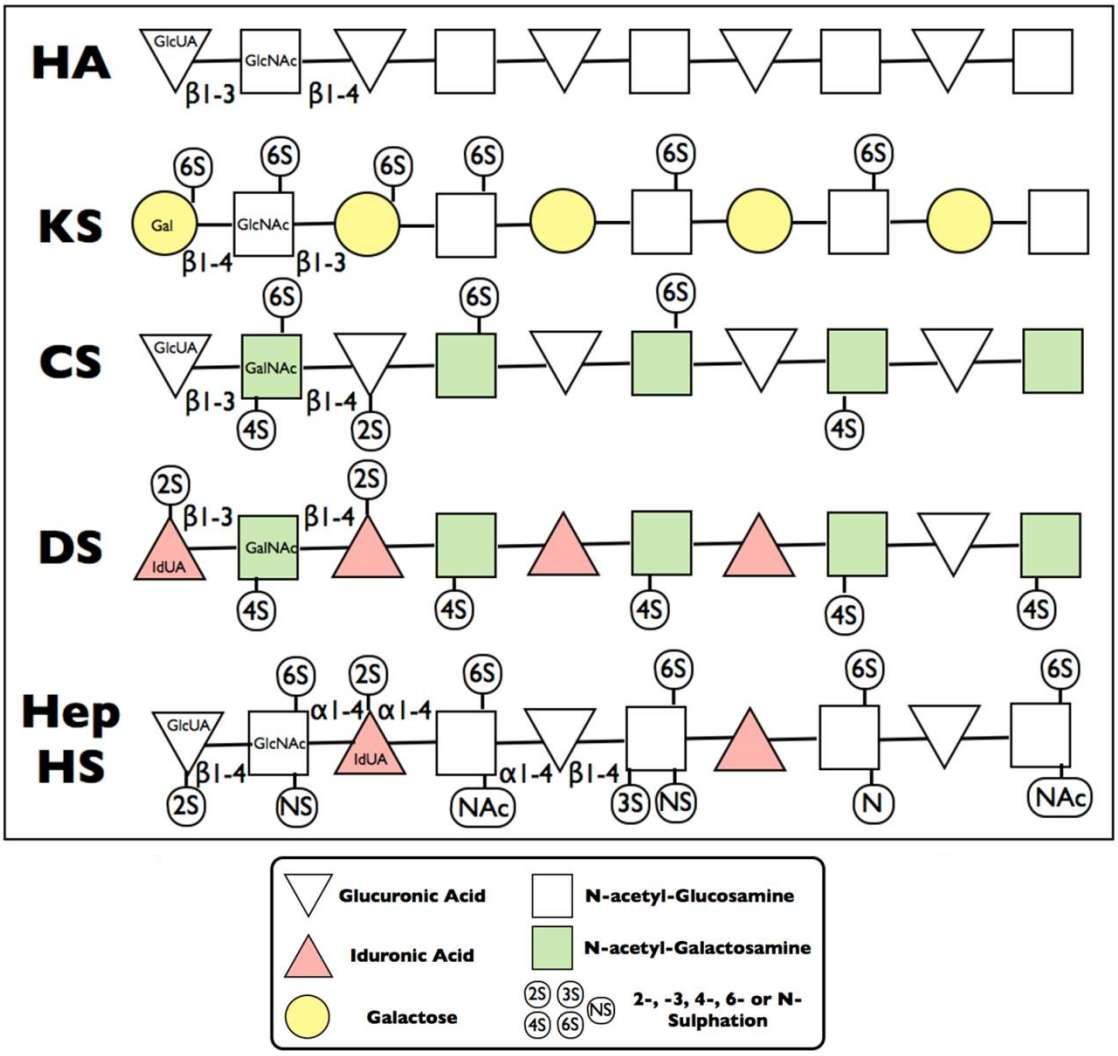


Figure 1.2 Diagram demonstrating the vast structural diversity of Glycosaminoglycan repeating disaccharide units (Reproduced with permission from Prof B Caterson)

GAG	Disaccharide Repeats	Pentasaccharide Glycan Combinations
HA	Glucuronic Acid & N-acetyl-Glucosamine	**2**
KS	Galactose & N-acetyl-Glucosamine	**64**
CS/DS	Glucuronic or Iduronic Acid & N-acetyl-Galactosamine	**1008**
Hep/HS	Glucuronic &/or Iduronic Acid & N-acetyl-Glucosamine	**2916**
Total		**3990**

Table 1.1 Disaccharide repeat composition & potential pentasaccharide glycan combinations for Hyaluronan (HA), Keratan Sulphate (KS), Chondroitin/Dermatan Sulphate (CS/DS) and Heparin/ Heparan Sulphate (Hep/HS) glycosaminoglycans (GAG).
(Reproduced with permission from Prof B Caterson)

1.13 Aggregating Proteoglycans

The aggregating proteoglycan family have been described as modular molecules, with integration of structural motifs (Fosang and Hardingham 1989). The two predominant aggregating proteoglycans are aggrecan and versican. Aggrecan is considered to be the benchmark of all proteoglycans and has been studied mainly in relation to its structural role and functional properties in cartilage, where it is the most abundant proteoglycan expressed (Paulsson and Heinegard 1984); (Hardingham and Fosang 1992).

1.14 Aggrecan

Aggrecan is a major component of cartilage and confers many of the physical properties of cartilage essential to its function. Aggrecan is a marker for cartilage and is expressed abundantly only in this tissue, but has been reported at low levels in other tissues. The aggrecan gene has been mapped to human chromosome 15q26.1 (Korenberg, Chen et al. 1993). The molecular weight of aggrecan is greater than 2500 kDa with 90% of its mass contributed to by around 100 CS and 50 KS chains, as well as 8-10 shorter N- or O- linked oligiosaccharides covalently linked to a core protein of 250-300 kDa (Vertel 1995).

The aggrecan core protein comprises of a series of disulphide-bonded domains that fold into two separate globular regions (G1 and G2) at the amino-terminus and a single globular domain (G3) at the carboxy-terminus (Roughley and Lee 1994). The G1 domain binds to HA and link protein within the ECM. The G2 domain is separated from G1 by a linear intergloblar domain (IGD) and does not contain any HA binding properties with its main function not fully understood. There is a large GAG substituted region consisting of KS and CS domains which extends between G2 and G3 at the carboxy-terminal of the core protein. However this region is highly variable between species. The final, G3 domain exhibits variation in its sequence compared to G1 and G2 domains due to alternative splicing. This domain comprises of a C-type lectin motif, a complement regulatory protein related motif, and one or two repeats of epidermal growth factor (Drickamer 1988). The human aggrecan core protein contains a KS attachment site and two CS attachment regions (Doege, Sasaki et al. 1987). During aging, changes in the size, number sulphation patterns and charge of KS and CS of aggrecan occur (Hardingham, Fosang et al. 1994). During the onset of osteoarthritis, catabolism of the aggrecan molecule takes place (Caterson, Flannery et al. 2000).

Within the ECM of articular cartilage, aggrecan forms enormous link-protein-stabilised aggregates with HA that are captured within the 3D-lattice work of type II collagen fibrils associated with other more minor collagen types and glycoproteins (Vertel 1995). The sulphated GAGs attached to aggrecan render the molecule as highly negative, attracting positive counter ions resulting in water retention, maintaining a highly hydrated tissue.

1.15 Versican

Versican is a large, CS-rich aggregating proteoglycan having a molecular weight in the excess of 1000 kDa, with the core protein being 400 kDa (Iozzo 1997). Versican has been found in cartilage, liver, intervertebral disc and skin. The human versican gene is located on chromosome 5, comprising of 15 exons encompassing over 90 kBases of genomic DNA (Iozzo, Naso et al. 1992). The structure of the versican protein core is considered to consist of several distinct domains, two extended regions and two globular domains present at the N-terminal end. The G1 domain is also known as the HA-binding site (Neame and Barry 1993). The number of GAGs covalently attached to the core protein of versican is between twelve to fifteen CS chains (Zimmerman and Ruoslahti, 1989).

1.16 Non-aggregating Proteoglycans

Non-aggregating proteoglycans do not have the ability to directly bind to HA. The family of non-aggregating proteoglycans include perlecan and the SLRPs decorin, biglycan and fibromodulin, characterised with the ability to interact with collagen (Roughley and Lee 1994).

1.17 Decorin

Decorin is present in cartilage throughout the life span. It has a molecular weight of around 120 kDa, comprised of 359 amino acids (Krusius and Ruoslahti 1986). The main unique feature of decorin is the eleven tandem repeats of leucine that contribute to the core protein molecular weight of between 36-45 kDa (Fisher, Termine et al. 1989). Human decorin usually carries one GAG chain (predominantly DS in non-mineralised tissues and CS in mineralised tissues) linked to the fifth amino acid residue sequence (Chopra, Pearson et al. 1985). In addition, avian decorin can exist in mono and biglycanted forms (Blaschke, Hedbom et al. 1996), and non-glycated forms have been isolated from adult cartilage (Johnstone, Markopoulos et al. 1993). The functional properties of decorin are its ability to interact with collagen fibrils, type I and II. It is able to influence both the rate of collagen fibrillogenesis and fibril diameter (Vogel and Trotter 1987).

1.18 Biglycan

Biglycan has an overall molecular weight of around 200 kDa with the core protein being between 37-45 kDa (Fisher, Hawkins et al. 1987). Human biglycan usually carries two GAG chains attached to serine 5 and 11, which may be DS or CS chains (Iozzo 1997) This proteoglycan can exist in a mono-glycanated form, i.e. carry one GAG chain, or none in a non-glycanated form in cartilage (Neame and Barry 1993);(Johnstone, Markopoulos et al. 1993). In cartilage, biglycan is present throughout life, however there has been evidence of age-related proteolytic processing. Biglycan is not thought to interact with collagen fibrils or directly influence fibrillogenesis with its precise role remaining unclear.

1.19 Fibromodulin

Fibromodulin has been described as a part-time proteoglycan that can also exist as a glycoprotein. It has a molecular weight between 38-45kDa and contains up to 5 KS GAG chains along the central domain of the core protein. Fibromodulin was first isolated from cartilage (Heinegard, Larsson et al. 1986), and exhibits some sequence homology to decorin and biglycan, although the amino terminus of the molecule contains a pair of tyrosine sulphate residues (Oldberg, Antonsson et al. 1989). Fibromodulin is a KS-containing proteoglycan and although the precise function of this molecule is unclear, it has been demonstrated to the have the capability of inhibiting collagen fibrillogenesis and binding to TGF-β (Plaas, Neame et al. 1990); (Hedbom and Heinegard 1993).

1.20 Collagens of Articular Cartilage

Collagen accounts for about two-thirds of the dry weight of adult articular cartilage. The tissue's material strength depends on the extensive cross-linking of the collagen and the apparent zonal changes in fibrillar architecture with tissue depth. Once laid down during development, there appears to be little capacity for articular chondrocytes to recapitulate the

overall collagen architecture if the mature tissue is injured or undergoes advanced degenerative changes.

Collagens and the large aggregating proteoglycans are the major constituents of the cartilage ECM. The collagen superfamily represent a diverse group of ECM molecules, that arise from multiple genes and differential splicing to give rise to a number of different collagen types. Collagens may be regarded as a family of proteins that, somewhere in their structure, contain three polypeptides forming at least one extended domain with a characteristic triple-stranded helix. They can subdivided into three classes, the fibrillar (types I, II, III, V and XI), non-fibrillar or basement membrane (types IV, VI, VII and XII), and fibril associated collagens with interrupted triple helices (FACIT) (Prockop and Kivirikko 1995). Overall, collagen represents 50-60% of dry weight articular cartilage and in adults, 90% of this consists of type II collagen (Kuettner 1992). In total, articular cartilage contains around ten different collagen types. Collagen types II, VI, IX, X and XI have long been known as major components of articular cartilage (Thomas, Ayad et al. 1994); (Bruckner and van der Rest 1994). However others have also been reported in low concentrations. These are types I, III, V, XII and XIV (Duance 1983); (Wotton and Duance 1994); (Eyre, Wu et al. 1987); (Watt, Lunstrum et al. 1992); (Duance, Crean et al. 1998). In mammalian articular cartilage, the primary collagen components are collagens II, IX and XI. The greatest quantitative difference occurs with maturation from the exclusively fine fibrils of young growth cartilages (approximately 10% collagen IX, 10% collagen XI, 80% collagen II) to the thicker and more varied fibril diameters of mature articular cartilage (approximately 1% collagen IX, 3% collagen XI, 90% collagen II) (Eyre, Wu et al. 1987).

1.21 Collagen Types II/IX/XI and the Heteropolymeric Template

Collagen type II is a fibril forming collagen secreted as a pro-collagen containing a large central triple-helical domain, linked to amino- and carboxy-terminal propeptides. Cleavage and removal of the propeptides by proteinases take place prior to fibril formation. Type II collagen is a homotrimer containing three identical ά chains and is produced by chondrocytes (Miller 1971). There are two forms of type II collagen that exist; Type IIA and type IIB and these are generated through alternative splicing of exon 2 that encodes a cysteine-rich domain (Ryan and Sandell 1990). Collagen type IIB is restricted to cartilage while type IIA is located in less mature tissue such as embryonic spinal ganglia (Sandell,

Morris et al. 1991). This collagen is susceptible to cleavage and degradation by matrix metalloproteinase 1 (MMP1), also known as collagenase 1, between resides Gly^{775} and Leu^{776} in the triple helix (Gadher, Eyre et al. 1988). Other MMPs that are also capable of cleaving type II collagen include MMP-8, -13 and -18.

Type XI collagen is another fibril forming collagen consisting of a large amino-terminal non-collagenous domain together with a 300nm triple helix. This collagen is a heterotrimer comprising of three different α-chains; α1, α2, α3 (Eyre, Wu et al. 1987). Type XI collagen forms an interconnecting secondary filamentous network that can provide links between fibrils (Bruckner and van der Rest 1994).

Type IX collagen is a family member of the fibril associated collagens with an interrupted triple helix (FACIT). This collagen is heterotrimeric and is only 190nm in length. It comprises of three triple-helical domains which are linked together by flexible regions as well as an amino terminal globular domain. Type IX collagen molecule consists of three collagenous domains and four non-collagenous domains with the later domains susceptible to proteolysis at a higher degree compared to the former. An interesting feature to this collagen is that it can also be perceived as a proteoglycan since the non-collagenous 3 domain contains a single site for attachment of a covalently bound CS chain (Huber, Winterhalter et al. 1988); (Vaughan, Winterhalter et al. 1985).

Within the ECM of articular cartilage, types II, IX and XI collagens form heterotypic fibrils and do not appear to alter dramatically in proportion between the different cartilage zones (Mendler, Eich-Bender et al. 1989). The exact spatial relationships, manner and temporal order of assembly of these different collagen types into heteromeric fibrils are not well understood. The basic structure of the fibrils is a four-dimensional-staggered polymer of collagen type II molecules heavily cross-linked head-to-tail by hydroxylysyl pyridinoline residues at the two telopeptide-to-helix sites. Collagen type IX decorates the surface of type II collagen fibrils, in particular the thin fibrils forming the chondron around the chondrocytes (Hagg, Bruckner et al. 1998). Seven cross-linking regions have been identified in the type IX collagen molecule and these interact with type II and IX collagen molecules (Diab, Wu et al. 1996); (Ichimura, Wu et al. 2000); (Wu, Woods et al. 1992). Collagen XI molecules are primarily cross-linked to each other in a head-to-tail manner and are thought to produce a template that constrains the lateral growth of the type II collagen hetero-fibril (Blaschke,

Eikenberry et al. 2000). Retained N-propeptide domains on type XI collagen are believed to inhibit fibril lateral growth (Gregory, Oxford et al. 2000).

1.22 Other Collagens

Type III collagen has been detected in samples of human articular cartilage (Aigner, Bertling et al. 1993); (Wotton and Duance 1994). This collagen has been found to be localised with type II collagen in the same banded fibrils and retaining its N-propeptide domain (Young, Gordon et al. 2000). It has been speculated that type III collagen is made in addition to type II by chondrocytes in response to ECM damage (Eyre, Weis et al. 2006). Type VI collagen has been found in cartilage localised to the pericellular environment of chondrocytes (Keene, Engvall et al. 1988). This collagen is a heterotrimeric molecule that is made up of three distinct chains. The main function of type VI collagen is to provide a protective fibrillar meshwork surrounding the cells. Type X collagen is also found in cartilage, mainly in the calcified zone in adult tissue and in the hypertrophic zone of the growth plate in developing bone (Linsenmayer, Chen et al. 1991). Type X is a homotrimer collagen molecule consisting three identical α1(X) chains with each containing a triple-helical domain with two non-collagenous regions. Types I, V, XII and XIV have also been shown to be present in low amounts in articular cartilage**.**

1.23 Other Extracellular Matrix Molecules

As well as proteoglycans and collagens, other components exist within the ECM of articular cartilage. These include elastin, fibronectin, osteonectin, cartilage intermediate layer protein (CILP), cartilage oligomeric matrix protein (COMP) and matrilins.

1.24 Kashin-Beck Disease

Several kinds of disabling polyarticular degenerative joint diseases occur with high frequency in remote parts of the world and are hardly known in the Western part of the world. One example is Kashin-Beck disease (KBD). KBD, also known as ‘Big Joint Disease’, is a

disabling disease of the bones and joints that leads to growth retardation and deformity of the joints. The disease affects people in a crescent shaped region through Tibet, northern China, Mongolia, Siberia, and North Korea. In China, about 30 million people live in areas where the disease is endemic, and at least 2-3 million people are estimated to be affected (Allander 1994); (Hinsenkamp 2001). The affected areas tend to be poor, rural and isolated from more developed urban cities. There is no cure, and no clear preventive measures exist, so prevalence can be as high as 80-90% in some small communities (Sudre and Mathieu 2001). KBD is a special endemic osteoarthropathy which starts in childhood, with symptoms occurring from the age of about 4 years. The disease affects the growth of joint cartilage, with the worst forms resulting in dwarfism, very short upper limbs, and deformed, painful joints with mobility as low as 30 degrees in the case of elbows. The most frequently affected joints are ankle, knee, wrist and elbow, leading to atrophied muscles (Moreno-Reyes, Suetens et al. 1998). Kashin-Beck disease was first identified in 1849 by a Russian doctor, Nikolai Ivanovich Kashin, but its causes remained unknown. The risk factors seem to include selenium deficiency in the soil, fungal contamination of barley (the staple grain), organic matter in the water, and iodine deficiency. These create an environment in which mycotoxins enter the rural food supply, but the precise cause is still subject to debate.

1.25 Pathology of KBD

The pathology of KBD has been demonstrated to occur in stages manifesting initial stiffness and swelling of finger and wrist joints in younger individuals, progressing into established osteoarthritis later in life with elbows, knees and ankles also largely affected (Sokoloff 1985). More recent radiological and physical observations of KBD patients from endemic areas of the Shaanxi province in China revealed a common set of symptoms which include:-

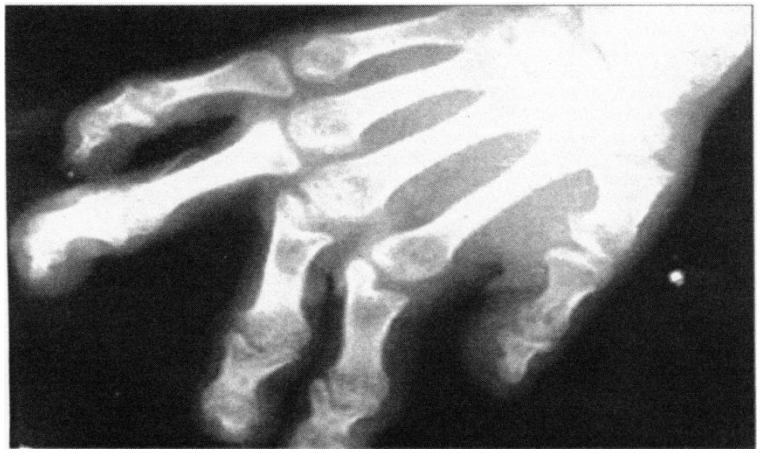
- shortening of the humerus and fingers
- enlargement of the interphalangeal joints
- shortened stature
- arthritic pain in the ankle and knee joints (minus swelling)
- restricted movement of these affected joints as well as the wrists and elbows.

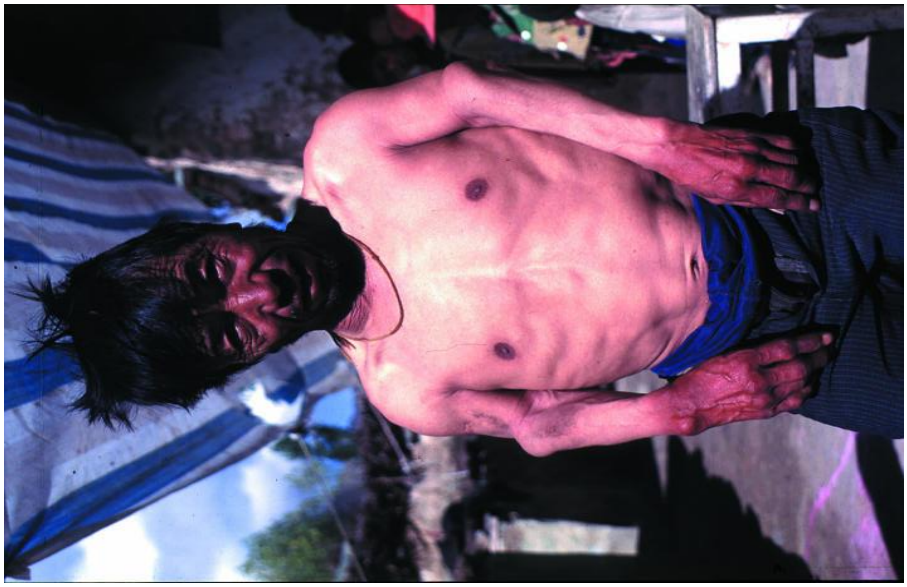
It has also been noted that the incidence of hand deformity is greater amongst children and generally adults appeared to manifest more symptoms. In addition, it has become apparent that distal joints including fingers, wrists and ankles are more severely affected that the proximal joints such as the hips and shoulders (Xiong 2001). Clinical photographs and X-rays demonstrating changes in KBD can be seen in Figure 1.3

1.26 Pathology of KBD; Cartilage and the ECM

Hyaline cartilage and the epiphyseal plate are the primary target of the disease. Pathological investigations on patients with KBD have revealed that the associated lesions in the osteochondral tissue predominantly occur at the epiphysis and articular cartilage at the ends of the tubular bones (Xiong 2001). The normal development and growth of these bones occurs at the growth plate by the process of endochondral ossification. This normally involves ECM synthesis, hypertrophy, matrix mineralisation and vascular invasion leading to apoptosis. This process allows for the normal homeostasis and the gradual replacement of chondrocytes with bone cells as the bone structure elongates (Ballock and O'Keefe 2003). The impairment of this process has been thought to be a major cause for the development of KBD. This has been attributed to the occurrence of articular and epiphyseal cartilaginous atrophy, throughout which chondronecrosis occurs accompanied by secondary changes (Xiong 2001).

Observations of the morphology of the cartilage derived from patients with KBD has revealed multiple necrotic lesions mainly located in the deep zones close to the bone and bone marrow, many appearing patchy, and differing in size (Pasteels, Liu et al. 2001). In the necrotic areas chondrocytes die and undergo degenerative changes which include loss of membrane organelles, swelling of the mitochondria and endoplasmic reticulum and nuclear deterioration (so-called cell ghosts). These cell ghosts disappear and the necrotic areas gradually become homogenised. In addition, amongst these necrotic regions there is also a loss of collagen fibrils and proteoglycans within the ECM (Pasteels, Liu et al. 2001). Following the death of chondrocytes, secondary changes occur. The surviving chondrocytes adjacent to the necrotic regions proliferate and form cell clusters with a high content of GAG within the ECM inside these clusters. Furthermore, chondrocytes within the clusters have many secretory vesicles and rough surface endoplasmic reticulum, showing pattern of secondary hyperfunction (Pasteels, Liu et al. 2001).





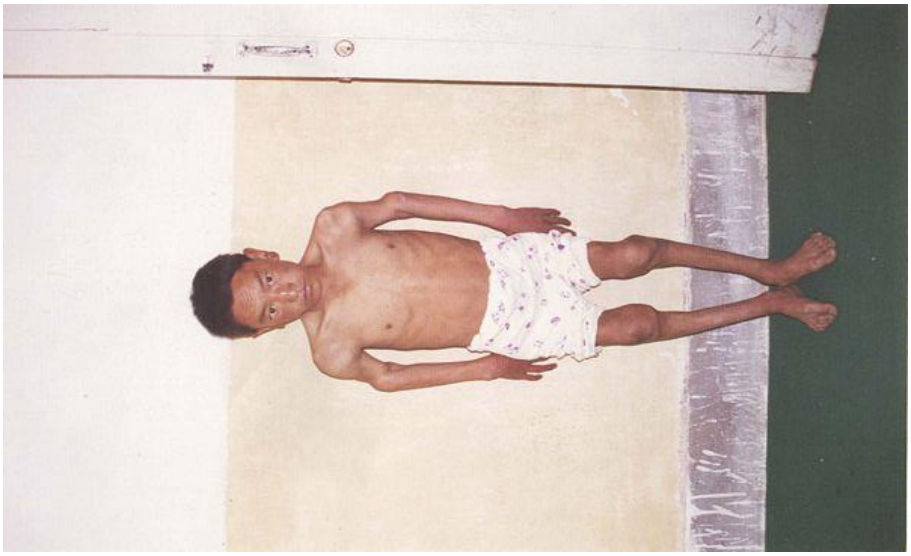


Figure 1.3: (a) Appearance of a child with KBD (b) Appearance of an adult with KBD

(c) Hand X-ray of an adult with KBD showing appearances not dis-similar to those seen in Rheumatoid arthritis (Hinsenkamp 2001)

As a direct result of these reactions, the processes of absorption, removal, organisation, dystrophic calcification and ossification gradually occur in the cartilaginous masses. Ultimately the necrotic regions heal with repaired scar bone tissue which eventually undergoes absorption and adaptation remodelling thereafter. As the normal epiphyseal plate is the growing centre of the bone, epiphyseal impairment can be followed by developmental disturbance which leads to short fingers (and toes), short limbs and even dwarfism. These pathological occurrences in the articular cartilage may progress slowly and secondary osteoarthritis with bone enlargement (osteophyte formation) and disfiguration of the joints can become prominent (Pasteels, Liu et al. 2001).

Following the examination of young KBD patients, further pathology issues have been discovered on examining supernumerary fingers. Histological findings have demonstrated a deficit of blood vessel penetration into the epiphyseal end plates (Pasteels, Liu et al. 2001) . The lack of blood vessels and epiphyseal bone formation has been seen in the phalanx end plate of the fingers, and this lack of vascularisation (an important step in normal endochondral ossification) can been seen as an initiator of KBD in some individuals. Possible explanations for the vascularisation deficit have been attributed to vasoconstriction of blood vessels in the peripheral joints of patients that endure cold winters (Pasteels, Liu et al. 2001). This has been suggested to be due to poor thyroid response to the cold due to deficiency in iodine and possibly selenium, as well as a vasoconstrictive effect of mycotoxins, or as a more specific effect of mycotoxins on epiphyseal ossification (Chasseur, Suetens et al. 1997); (Moreno-Reyes, Suetens et al. 1998). Using these sources of evidence, it can be concluded that articular cartilaginous atrophy as well as reduction in vascular penetration, resulting in disturbed processes at the distal joints, could account for the occurrence of KBD.

Recent studies have made comparisons of the pathology of cartilage derived from patients with KBD compared to those with other joint diseases such as osteoarthritis. Analysis of the tissue and serum from children and adults suffering from KBD has been shown to display many similarities as well as differences to patients suffering from other joint disease (such as osteoarthritis). H&E and Toluidine blue staining of articular cartilage sections obtained from KBD patients demonstrated cellular necrosis and decreased proteoglycan content in addition to surface fibrillation similar to that seen in different stages of degenerative joint disease progression (Cao, Li et al. 2008). In the same study it was noted that in general the occurrence of cellular necrosis occurred in patches above the subchondral bone, whereas diminished extracellular matrix staining was present throughout all

morphological zones of cartilage obtained from KBD patients. This finding is in contrast with that found in osteoarthritic cartilage, where a progressive degeneration from the surface to the deeper zones is apparent (Slater, Bayliss et al. 1995). Further studies have compared the expression of CD44 in cartilage from KBD patients. CD44 is a membrane glycoprotein that is very important for cell-matrix interactions and proteoglycan retention around chondrocytes, and it has been suggested that alteration of CD44 metabolism occurs during joint disease such as osteoarthritis (Ostergaard, Salter et al. 1997); (Takagi, Okamoto et al. 2001). The expression of CD44 in articular cartilage from KBD patients (adults and children) indicated detection around the periphery of chondrocyte sections, whereas in normal child cartilage the detection of CD44 was minimal or absence (Guo, Zuo et al. 2006). This study suggested that the up-regulation in CD44 expression in KBD could be indicative of increased cartilage matrix metabolism in the disease. This result was found to be similar in other joint diseases such as osteoarthritis where there is an increase in CD44 expression in the cartilage and synovial fluid (Fuchs, Dankbar et al. 2004); (Fuchs, Rolauffs et al. 2003). The presence of aggrecanase-generated catabolic neoeptitopes or aggrecan catabolites in KBD cartilage has also been investigated (Cao, Li et al. 2008). Many proteolytic enzymes such as aggrecanases have been shown to be involved in cartilage extracellular matrix degradation, and aggrecanase-mediated aggrecan degeneration has been shown to occur during osteoarthritis (Lark et al, 1997; Huang, 2008). It has been demonstrated that this also occurs in cartilage derived from KBD patients, with proteolytic cleavage within the IGD of aggrecan by aggrecanases (Cao, Li et al. 2008). Furthermore, the involvement of both IL-1β and TNF-α has been demonstrated in the pathogenesis of KBD as well as during osteoarthritis (Cao, Li et al. 2008).

1.27 Aetiology of KBD

The aetiology of KBD remains controversial. Scientific literature is dominated by three hypotheses based on respectively selenium (Se) deficiency, mycotoxins from contaminated storage grains, and organic matter (fulvic acid) in drinking water.

1.28 Selenium Deficiency

Selenium (Se) deficiency has been suggested as a risk factor for KBD since concentrations in the serum of subjects living in areas where the disease is endemic, and in the food they eat, are lower that the respective values in areas without endemic disease (Wu and Xu 1987); (Yang and Xia 1995). A geographical association between areas of low Se status and the occurrence of KBD was first noted in the 1970s. In addition, no reports of the disease have been recorded in areas with Se rich regions (Ge and Yang 1993). Geographical studies have linked depleted soil Se levels and the presence of KBD across China (Figure 1.4). Furthermore, a temperate and humid environment has been associated with low Se levels, resulting in a specific area of reduced levels in the soil in China occurring from north-east to south-west. This pattern correlates with endemic areas for KBD. The hypothesis behind this correlation is that low Se soil levels results in reduced Se concentrations present in food grains, vegetables and drinking water, therefore causing a Se deficient diet (Tan, Zhu et al. 2002). Fluorimetric analysis studies of the drinking water and cereals from the KBD affected areas show a much lower Se content compared to non-affected areas (Pasteels, Liu et al. 2001). In addition, Se content in the hair and blood of individuals suffering from KBD correlated with the low Se concentrations in food grains around the endemic areas (Zhang et al. 2001). The average intake of individuals in the affected areas has been shown to be around 3-5µg/day compared to approximately 10-11µg/day in non-affected regions (Sun, Li et al. 2012). Selenium is an essential trace element for humans, animals and some species of micro-organisms (Pinsent 1954);(Oldfield 1987).

In the majority of cases, the Se atom exists as a selenocysteine residue of the protein polypeptide chain and has an important role in maintaining efficient catalysis. Selenium is an integral part of the enzyme glutathione peroxidise (GPx) in man and animals (Rotruck, Pope et al. 1973). This enzyme can protect the organism against oxidative damage by reducing lipoperoxides and hydrogen peroxide (Ebert-Dumig, Seufert et al. 1999). GPx exists in chondrocytes and removes intracellular H_2O_2 preventing the formation of other reactive oxygen species such as the hydroxyl radical (Baker, Feigan et al. 1988). GPx also functions to decrease organoperoxides that form from lipid peroxidation such as fatty acid hydroperoxides (Imai and Nakagawa 2003). Another important family of enzymes reliant on Se are deiodinases (types I, II, III). Types I and II function to catalyse the activation of prohormone thyoxine (T4) to the active thyroid hormone 3,3',5-triiodothyronine (T3), and

Figure 1.4: Map demonstrating the distribution of two Se-responsive diseases; KBD and Keshan disease (a cardiac disease) across China. The distribution correlates very closely with areas of Selenium deficiency in soil.

type III deiodinates T4 and T3 to their respective hormonally inactive metabolites (Kohrle 2000). Selenium is also incorporated into other enzymes or proteins, such as phospholipid hydroperoxide, selenoprotein P (Se-p), which all have important human health functions (Tan, Zhu et al. 2002).

Low Se status has been shown to correlate with chondrocyte membrane/ DNA rupture in individuals that suffer with KBD indicating that a deficiency in Se levels could be a cause for chondrocyte damage. However, how this occurs it not fully understood (Li, Duan et al. 1990). Nevertheless, it can be postulated that knowing the function of GPx's to act as antioxidants, the deficiency in Se levels causes reduction in the enzyme levels resulting in increased oxidative damage (lipid peroxidation / DNA damage) within chondrocytes. *In vitro* studies have also provided information about the possible effect of a diet deficient in Se on chondrocytes. For instance, human embryonic cartilage cells cultured in the presence of Se could prevent damage by organic matters, increase the activity of GPx and decrease the

production of lipid peroxide (Peng and Yang 1991). Furthermore, a study investigating the effect of nivalenol (a mycotoxin commonly found in soil fungi and in cereals grown in the regions where KBD is prevalent), on cultured primary rabbit chondrocytes in the presence and absence of selenium, found in a dose dependent manner that nivalenol reduced the production of total protein, impaired DNA synthesis and reduced proteoglycan metabolism. With the addition of selenium, protein content was reported to be higher and proteoglycan metabolism was improved (Chu and Cao 2003).

Animal studies have also provided information about potential correlation between the effects of low Se levels and the pathology of KBD. Studies have provided evidence that a deficiency in Se is a cause of disrupted endochondral ossification and bone growth, which occurs in the pathology of KBD. Electron-microscopic investigations carried out on Se deficient rats demonstrated chondrocyte endoplasmic swelling, nucleus degeneration and cellular necrosis. This resulted in decreased blood volume and mineral density of the developing bones. From these studies it was suggested, due to chondrocyte cell death, there is impaired bone metabolism which is a pathological process that is known to occur in KBD (Sasaki, Iwata et al. 1994). Further studies carried out on Se deficient rats also showed retarded bone growth and a reduction in bone calcification correlating to abnormal biomechanical properties of the developing bone (Moreno-Reyes, Egrise et al. 2001). Following bone bending tests, young rabbits fed a selenium deficient diet demonstrated a significant decrease in biomechanical strength of the bones, while the bones belonging to the control group always had the largest modulus of elasticity (Turan, Balcik et al. 1997). Animal models have also suggested a role for a lack of Se and reduced ECM production. Reduced sulpho-transferences and alkyl phosphatase concentrations in Se deficient rats may be a cause for the decreased presence of proteoglycans within the bones and cartilage since these enzymes play an important role in the synthesis of GAG chains (Sasaki, Iwata et al. 1994). Furthermore, fulvic acid and selenium deficiency in mice have been reported to be associated with degeneration of articular cartilage, modification of type I collagen in bone and type II collagen in cartilage, development of fibrocartilage at the articular surface as well as impaired formation of subchondral bone (Yang, Wolf et al. 1993). Collectively, these animals studies indicate that Se deficiency can result in chondrocyte death, altered ECM production, abnormal bone mineralisation as well as impaired endochondral ossification.

Overall, these epidemiological and biochemical studies provide evidence that the lack of Se is associated with the development and pathology of KBD. However, it has been noted that some unaffected individuals live within endemic areas and are exposed to a Se deficient

diet (Peng, Yang et al. 1992). Therefore, this suggests that other aetiological factors are important in the onset and pathological development of KBD.

1.29 Iodine Deficiency

In China, endemic selenium deficiency is closely related to endemic iodine deficiency (Moreno-Reyes, Suetens et al. 2001). The existence of an iodine deficiency disorder known as goitre has been associated with KBD patients, compared to unaffected individuals (Moreno-Reyes, Suetens et al. 1998). Furthermore, hypothyroidism secondary to iodine deficiency is found significantly more often in villages affected by KBD compared to control villages (Moreno-Reyes, Suetens et al. 2001). Hypothyroidism impairs skeletal development in children by causing epiphyseal dysgenesis, delay of osseous development and reduced endochondral ossification (Delange, Camus et al. 1972); (Moreno-Reyes, Suetens et al. 2001). Therefore it is possible that iodine deficiency and KBD pathology may be associated. A physiological function of iodine is its involvement in thyroid hormones (T3 and 4) and subsequent metabolism (Yang, Ge et al. 1988); (Ge and Yang 1993). Therefore, it can be postulated that in a similar way to selenium, iodine deficiency may influence thyroid hormone T4 and then T3 (active form of T4) potentially affecting bone metabolism. However, studies carried out on children demonstrated that low hair selenium concentration and presence of fungal cereal contamination was significantly associated with an increase of KBD, but low urine iodine was not (Zhang, Neve et al. 2001), indicating that the progression of KBD has been seen to occur without iodine deficiency.

1.30 Organic matter intake

Increased levels of organic matter known as humic substance has been noted to be present in the drinking water supply in KBD endemic regions compared to unaffected areas (La Grange, Mathieu et al. 2001); (Peng, Wang et al. 1999). Humic substance is ubiquitous in water, soil, coal and other sources. It is a heterogeneous high-molecular-weight organic compound, and its composition varies in different geographic regions. According to its solubility in aqueous acids or bases, humic substances are generally classified into three fractions – humic acid, fulvic acid (FA) and humin. Humic acid is precipitated when the pH

condition is lowered to below 2, whereas FA is soluble under all pH conditions (Aiken et al, 1985); (Liang, Tsai et al. 1999). In addition, humic acid and FA comprise mainly of complex hydrophilic polyelectrolytic polymers of benzene rings with various substitutions such as carboxyl, carbonyl, methoxyl, hydroxyl and phenol groups. The polymerized phenols that comprise humic acid and FA are derived from rotten vegetation and have been reported to cause brownish colour to natural waters (Sokoloff 1988). Because humic substances play an important role in binding and fate of inorganic and organic compounds in the natural environment, there have been many studies on the effect of these substances on the behaviour of inorganic and organic pollutants (Liang, Tsai et al. 1999). In recent decades, it has been found that humic substances are also related to human health. The majority of the biological studies carried out on the effect of humic substances have focused more on FA rather than humic acid.

In vitro studies investigating the effect of FA on human embryonic articular chondrocytes observed elevated levels of lipid peroxidation which was found to be induced by reactive oxygen species (ROS). Humic acid has been demonstrated to have similar effects on rabbit articular chondrocytes since cell lysis and elevated concentrations of intracellular calcium were apparent, both of which indicate the occurrence of biomembrane damage (Liang, Tsai et al. 1999). ROS can induce damage to cell membrane structures and to intracellular DNA, RNA and proteins which ultimately influence cellular functions and lead to cell necrosis (Peng, Yang et al. 1992). In addition, there was an increase in lipid peroxidation in the blood and liver of rats that were fed normal diets and received water containing FA from the KBD region (Peng, Wang et al. 1999). It has also been found that FA can be incorporated into bone and cartilage of rats, the target tissues of KBD, where the selenium concentration was low (Yang, Niu et al. 1993). Therefore, from these studies it can been suggested that FA and humic acid promote oxidative stress, and as a result cause damage to chondrocytes potentially resulting in cell death, a feature seen in the progression of KBD (chondronecrosis).

Humic substances have also been shown to affect ECM components associated with cartilage. In chick embryos, FA was found to inhibit the conversion of procollagen II to collagen II and resulted in the structural alteration of collagen type II (Yang, Niu et al. 1993). Morphological studies on KBD affected tissues show a distorted deposition of collagen fibres in cartilage. Molecular defects in collagen II have also been seen in the articular cartilage of KBD patients demonstrating over-modification and impaired conversion of pro-collagen II to collagen II (Yang, Bodo et al. 1991). Furthermore, FA has been shown to inhibit the activity

of galactosyl-hydroxylysylglucosyl-transferase and further disturb the post-translational modification of intracellular collagen in mice (Zhu, Zhu et al. 2004). Therefore, from this it can be suggested that humic substances, in particular FA, may play an important pathological role in the abnormality of collagen present in the cartilage of KBD patients.

Concentrating on the KBD-related pathology of proteoglycans, the presence of ROS may play an important role. The production of hydrogen peroxide and the superoxide radicals damage proteoglycans at the protein core level resulting in them being smaller in size than normal (Panasyuk, Frati et al. 1994). A GAG component, such as HA, has also been shown to decompose following incubation with ROS (Yamazaki, Fukuda et al. 2003), however, whole GAG chains have shown resistance to the radicals (Panasyuk, Frati et al. 1994). The reduction of matrix proteoglycans may have a structural affect upon the ECM as well as potentially influencing signalling within the ECM (Kresse and Schonherr 2001). Although humic substances have not been used in these described proteoglycan studies, knowing that they are a source of reactive radicals can provide hypothesis that FA or humic acid may induce these effects on proteoglycans within the ECM and contribute to the pathology seen in KBD patients. Studies need to be carried out to confirm this.

1.31 Mycotoxin Intake

Prolonged exposure to humid conditions is thought to make grains more susceptible to mould as seen in barley grains around KBD endemic areas of Tibet (Haubruge, Chasseur et al. 2001). The presence of fungal mycotoxins has been shown to contribute to an increase in the content of semiquinolone radicals in cereals. A statistically significant link has been established between cereal contamination in some species of fungi and the incidence of KBD (Chasseur, Suetens et al. 1997). Certain classes of fungi have been identified to contaminate the grain among various areas affected by KBD is China. Prevalent types of fungi include *Fusarium* (*F*) *oxysporum*, *F. moniliforme*, *F. graminearum* and *Alternaria alternate keissler*. The mycotoxins produced by these fungal species are known to include deoxynivalenol (DON), T-2 Toxin, nivalenol (NIV), butenolide (BUT), moniliformin (MON) and alternariol methyl ether.

Some histopathological observations made on patients suffering from KBD are similar to those seen in experimental mycotoxin administration. Feeding chickens with fodder containing fusarium grain from KBD areas caused obvious retrograde affects in the growth

plate of the knee joints. The chondrocytes showed growth hindrance with degenerative changes as well as a band-shaped necrosis in the growth cartilage (Cao et al, 1998). It has also been reported that skeletal defects in pregnant mice are induced by the administration of mycotoxins (Debouck, Haubruge et al. 2001) as well as inducing delayed ossification in the chick (Bergsjo, Herstad et al. 1993). Human embryonic articular cartilages exposed to an aqueous solution of mycotoxins extracted from *F. oxysporum* demonstrated increased levels of lipid perioxidation and biomembrane damage. In addition, following further examination of this aqueous solution of mycotoxins, high levels of reactive radicals were found (Peng, Yang et al. 1992). These findings could imply that similarly to FA and humic acid, mycotoxins are sources of radicals that induce oxidative cell damage. Mycotoxins, such as NIV, can also affect active proliferating chondrocytes by inhibiting the synthesis of DNA, causing DNA breakage and inducing DNA to mutate. It also suppresses the synthesis of protein by interfering with the function centre of the peptidyl transferase of ribosomes, and inhibits the initiative and the terminal reaction (Tsuda, Kosaka et al. 1998).

Previous studies have shown that mycotoxins can also influence ECM production. Chicken embryo chondrocytes incubated with mycotoxins have been shown to produce more levels of collagen type I than type II (Wang, Xu et al. 1991), which may contribute to the secondary effects seen on bone during KBD pathology. Mycotoxins, such as T-2 toxin and NIV, have been demonstrated to disturb proteoglycan metabolism in chondrocytes cultured in vitro, by decreasing synthesis (Cao et al 1998), which may play a pivotal role during pathogenesis of KBD. Furthermore, mycotoxins can inhibit aggrecan synthesis in chondrocytes and promote aggrecanases and aggrecan degradation. All of these effects will upset the balance between aggrecan synthesis and degradation. This induces aggrecan loss from cartilage, which may be the initiation of the cartilage degradation (Li, Cao et al. 2008). In addition, if mycotoxins are sources of radicals (as with humic substances) this could also cause damage to the ECM composition by a similar method. Furthermore, mycotoxins can also influence the secretion of cytokines which may also affect ECM components. Mycotoxins, DON, NIV and T-2 toxin have been demonstrated to superinduce IL-2, IL-4 and IL-5 gene expression in T-cells (Ouyang, Azcona-Olivera et al. 1995). Mycotoxins, such as T-2 toxin have also been experimentally shown to induce IL-1β and TNF-α expression in chondrocytes (Li, Cao et al. 2008). These cytokines are known to be structurally and functionally involved in the metabolism of ECM components associated with target tissue of KBD, such as cartilage aggrecan and collagen type II. Moreover, it has been confirmed that levels of IL-1β and TNF-α are increased in adult KBD patients' synovial fluid (Yang, Wang

et al. 2001). These cytokines may go on and enhance other factors such as matrix metalloproteinases from chondrocytes and inhibit the normal synthesis of cartilage components such as collagen type II and IX and promote the abnormal secretion of collagen type I. Furthermore, through the up-regulation of pro-inflammatory cytokines, this can further contribute to ECM breakdown such as cartilage aggrecan degradation. From these hypotheses, it can be suggested that the actions of mycotoxins may contribute to the abnormal synthesis and elevated degradation of the cartilage ECM and degeneration of chondrocytes as seen in the pathology of KBD. However, more studies need to be carried out to confirm this.

Overall, in considering each of the aetiological factors demonstrated to have a potential to contribute to the pathology initiation and progression of KBD, the sources of evidence have suggested that the aetiology of KBD is multifactorial and it is likely to involve all described environmental factors.

The mycotoxin used in this study was Nivalenol, it has the chemical formula $C_{15}H_{20}O_7$, and its molecular structure is shown in Figure 1.5.

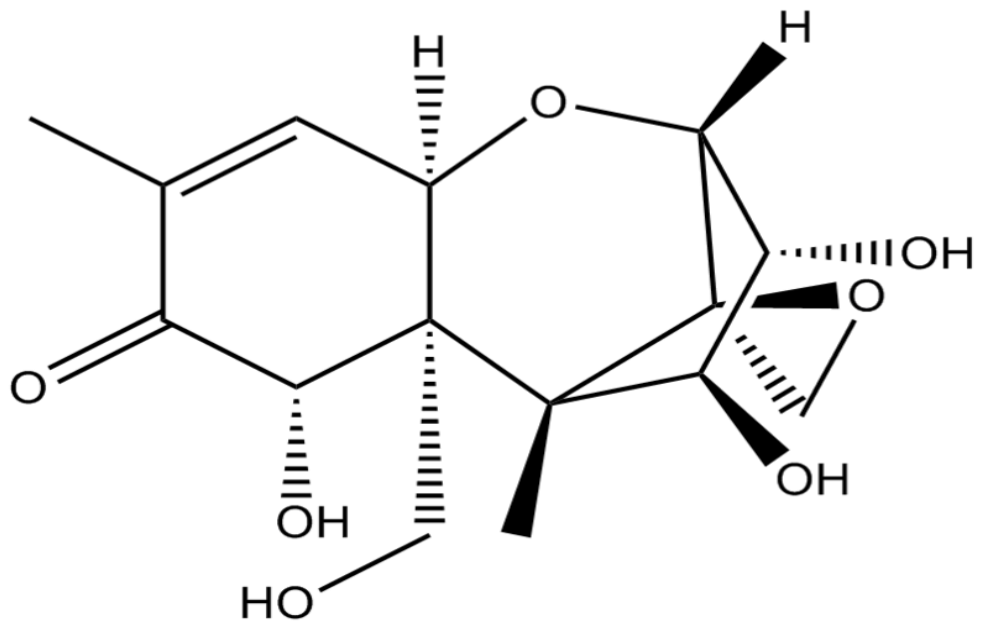


Figure 1.5: The molecular structure of the mycotoxin Nivalenol

1.32 Current Treatments of KBD

Several attempts have been made to improve or relieve some of the osteoarthritic symptoms associated with KBD. The most common attempt has been Se supplements, with or without additional antioxidant therapy (vitamin E and vitamin C). In some studies this has been reported to be successful, but in others no significant improvement could be seen compared to control groups. In addition, KBD animal model studies have been used to further show the efficacy of Se supplementation as a source of treatment. It has been demonstrated that rats fed Se supplemented water show a decreased concentration of radicals in the bone when compared to rats that did not receive Se supplemented water (Peng, Wang et al. 1999). In another study it was also shown that sodium selenite supplementation in rats fed on cereals and water from KBD areas improved the adverse effects on cartilage, decreasing the collagen I to II ratio (Wang, Xu et al. 1991). Interestingly, KBD patients receiving supplementation of sodium selenite have also demonstrated positive effects (Ge and Yang 1993). However, major drawbacks of selenium supplementation are logistic difficulties, potential toxicity and low compliance. Iodine supplementation has been tested and also compared to the effects of Se addition. A study demonstrated that KBD patients taking iodinised oil displayed improved height, and extra Se intake did not confer any added beneficial effects. Thus it was concluded that iodine, as well as Se could provide positive therapy for KBD (Moreno-Reyes, Mathieu et al. 2003). In reflection, it may be worth advocating these measures to prevent the onset of KBD, rather than using them as treatments in established cases.

With the mycotoxin theory in mind, packing of grains before storage was proposed in the Guanxhi Province, but results are not reported in the literature. However, changing the grain source has been reported to be effective in Heilongjang Province and North-Korea. With respect to the role of drinking water in KBD affected areas, changing water sources to deep well water has been reported to decrease the X-ray metaphyseal detection rate in different settings. Again this would be a method of prevention rather than cure.

An approach to the treatment of established KBD has been the use of physical applications and surgical procedures. Physical therapy can have significant effects on joint mobility and joint pain in KBD patients. By implicating massaging and regular, appropriate exercise, its aim has been to improve joint mobility and pain before the onset of major abnormalities. This has proven to be a success in studies performed in Tibet (Mathieu,

Suetens et al. 2001). In more extreme cases surgery has been a treatment option. Surgical corrections have been made a success by Chinese and Russian orthopaedic surgeons. The surgery usually consists of correction of axial and rotational deformities just beneath the growth plate and this has been shown to retain a lot of the normal knee mobility as well as improving pelvic, spine and patellar function. As a direct result, KBD patients have shown to become more independent proving this form of treatment as a very successful but invasive procedure (Liu, Wang et al. 1998).

1.33 Aims

The above review has highlighted the importance of the ECM components, such as proteoglycans, GAG and collagens in cartilage tissue formation and maintenance. Furthermore, it is evident that KBD is complex and multifactorial disease, and that certain environmental contributors (predominantly selenium deficiency, iodine deficiency, organic matter intake and mycotoxin intake) are acknowledged as prime causative factors in the disease process. With this background in mind, the aims of this thesis were to investigate the potential influences of some environmental factors (selenium and the mycotoxin Nivalenol) on an *in vitro* articular cartilage model that mimics certain pathological aspects of KBD. Continuing from these studies, attempts were made to relate observed effects with those associated with cartilage tissue destruction and changes during KBD. We hypothesise that growth and metabolism of cartilage will be affected by exposure to either selenium or the mycotoxin Nivalenol or both in combination and these effects will mimic those found in KBD.

The specific aims to test this hypothesis were:

Aim 1: To develop an *in vitro* culture system that mimics cartilage matrix metabolism pathology in KBD.

Aim 2: To investigate the histological effects of selenium and NIV on cartilage matrix metabolism.

Aim 3: To investigate the biochemical effects of different sources of selenium and NIV on cartilage matrix metabolism.

These results will contribute to the better understanding of the biological mechanisms associated with cartilage pathology related to KBD and the potential roles that etiological factors, selenium and NIV play in KBD pathogenesis.

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 Materials

The majority of chemicals and materials utilized were obtained from Sigma-Aldrich Chemical Corporation, UK, including Selenium-l-Methionine, Sodium selenite and Nivalenol. For those that were not, the supplier will be stated.

Pronase (7 units/ml) from Streptomyces griseus was obtained from Roche Diagnostics, Collagenase Type II (100u/ml) prepared from Clostridium Histolyticum was obtained from Lorne labs, Worthington Biomedical Corporation, UK. Dulbecco's Modified Eagles Medium (DMEM) without Sodium Pyruvate with 4500mg/ml Glucose with pyridoxine, Gentamycin (1000x stock) and heat inactivated Foetal Bovine Serum (FBS) were obtained from Invitrogen, UK. Biopore filter inserts (12 mm, 0.4µm pores) were obtained from Millipore, Bedford, MA. Recombinant human TGFβ_2 was obtained from Peprotech, UK. 40µm Cell strainers were obtained from BD Falcon, Belgium. Phosphate Buffered Saline (PBS) tablets were obtained from Oxoid. Tissue culture plates were obtained from Corning. Histobond microscope slides for both histology and immune-labelling were obtained from RA Lamb Ltd, Eastbourne, UK. For Immuno-labelling analysis Cryo-protect/Cryo-M-Bed tissue mountant was obtained from Bright/UK. Keratanase was obtained from AMS Biotechnology, UK. Propidium Iodide (0.5µg/ml) Molecular probe was obtained from Invitrogen, UK and Vectorsheild was obtained from Vector Laboratories Ltd, UK. Mayers haematoxylin and Eosin (Aqueous 1%) were obtained from RA Lamb Ltd, Eastbourne, UK. The 96 well multiplates were obtained from Elkay Laboratory products UK, Ltd, Basingstoke, UK. Powdered DMMB was obtained from Serva. Formic acid was from BDH. Lactate assay kit was obtained from Trinity Biotech. The Combiplate 8 Eliza plates were obtained from Thermoelectron Corporation. Ethylenediaminetetraacetic Acid Disodium Salt Dehydrate, Acetic Acid, Xylene, Ethanol, sodium hydroxide, hydrochloric acid, perchloric acid, dialysis tubing and propan-2-ol were all obtained from Fisher.

2.2 Preparation of scaffold free neo-cartilage graft production utilising an *in vitro* Transwell culture system- Isolation of articular chondrocytes

A modification of the method described by Kandel et al, 1995 was used to engineer the cartilage grafts. Articular cartilage shavings were harvested from 7 day bovine metatarso-phalangeal and/or metacarpo-phalangeal joints (See Fig 1). Full depth cartilage was harvested and chondrocytes isolated using well-established aseptic techniques. Cartilage was digested in 7 units/ml pronase in Dulbecco's modified Eagle medium (DMEM) containing 50µg/ml gentamycin and 5% (v/v) heat inactivated FBS for 3 hours at 37°C. The cartilage shavings were then washed with DMEM containing 50µg/ml gentamycin, and further digested in 100 units/ml collagenase type II in DMEM containing 50µg/ml gentamycin and 5% (v/v) heat inactivated FBS for more than 12 hours at 37°C.

The digest was filtered through a cell strainer and the cell suspension was centrifuged at 1800 rpm for 10 minutes. The supernatant was removed and the chondrocyte pellet was re-suspended in 10ml DMEM containing 50µg/ml gentamycin, gently mixed and then made up to 30mls with the same DMEM solution. These dilutions were accounted for in the final cell count calculation.

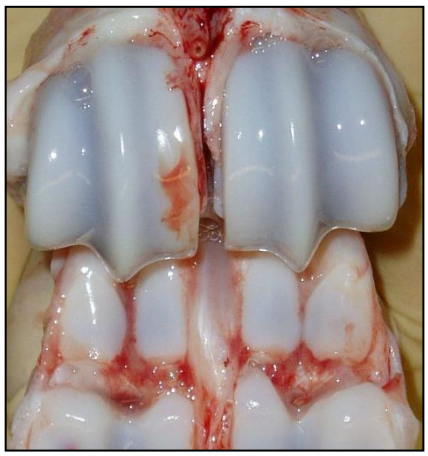


Figure 2.1 Exposed bovine metatarso-phalangeal joint

2.3 Cell Count

The cells were counted using a Sigma bright-line haemocytometer and a bright field microscope. If the cell yield was high making cell counting difficult and inaccurate, then cell

suspension was diluted with PBS prior to the cell count. This dilution factor was then taken into account when calculating the final cell numbers.

The 30ml cell solution (above) was gently mixed and 10µl was taken and placed into an Eppendorf tube with 190µl of DMEM solution added. A cell count was then performed. The subsequent cell concentration per ml (and the total number of cells), were determined using the following calculations:

- **Cells per ml =** the **average** count of cells per square on haemocytometer (5 squares counted in total, centre square and 4 corner squares on haemocytometer grid) **x** dilution factor (i.e. 20 unless further dilution with PBS used) **x** 10^4.

- **Total cells** = Cells per ml **x** original volume of fluid from which the sample was removed (i.e. 30ml)

With the total cell count established, the cell solution was again centrifuged at 1800rpm for 10mins. The supernatant was removed and the chondrocyte pellet was re-suspended in DMEM containing 10% FBS and 50µg/ml gentamycin of sufficient volume to give a cell concentration of $10x10^6$ cells per ml. In all the following experiments, cells were seeded at $4x10^6$cells per well unless otherwise stated (i.e. 400µl of solution per well).

2.4 Seeding chondrocytes onto Millipore filter inserts and culture maintenance using a Transwell culture system

In the following experiments, unless otherwise stated, cells were seeded at $4x10^6$ cells/400µl onto 0.6cm^2 Millicell^{CM} filter inserts (0.4µm pores) pre-coated with 40µl of 0.5mg/ml chick type II collagen, and each filter insert positioned into a well of a 24 well tissue culture plate (**Figure 2.2**). Before seeding each coated insert was washed 3 times with serum free DMEM.

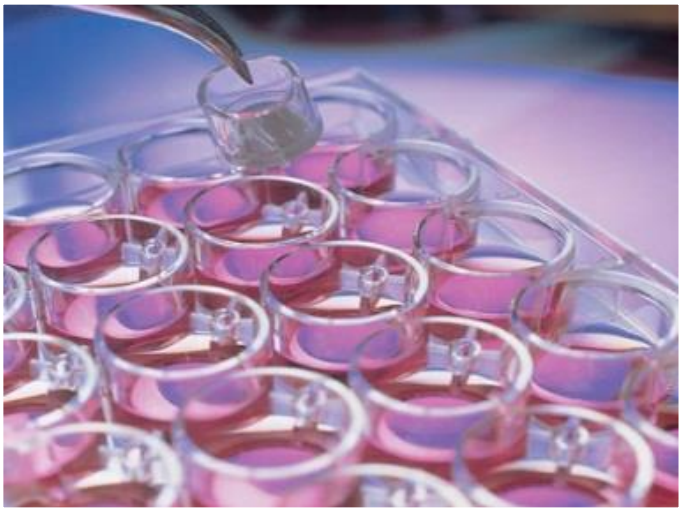


Figure 2.2. Schematic depicting the positioning of a millipore filter insert into a 24 well culture plate.

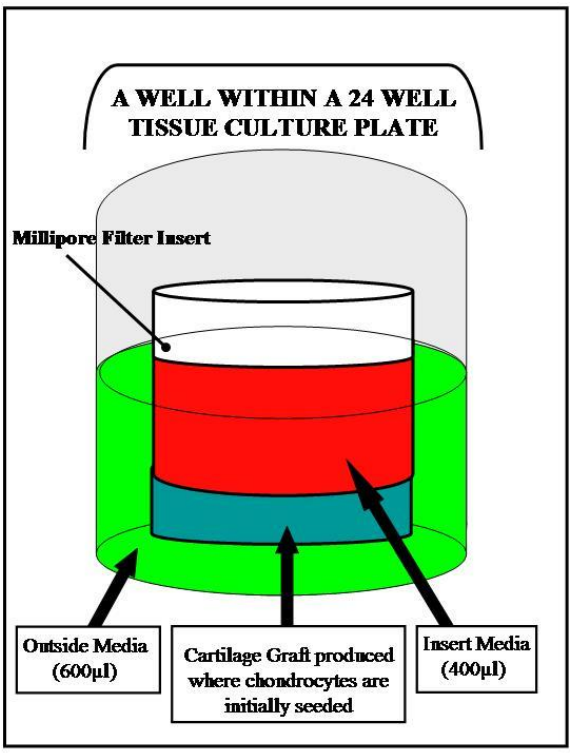


Figure 2.3 A Transwell culture system used to generate neo-cartilage grafts

A schematic of the compartmental organisation of a Transwell culture system is shown in **Figure 2.3**. Outside of the filter insert, within the well of the culture plate, 600µl DMEM containing 50µg/ml gentamycin and 20% FBS was pipetted. The seeded chondrocytes were left to settle for 48 hours at 37°C in a humidified atmosphere containing 5% CO_2.

2.5 Culture Maintenance and establishment of a Transwell culture system to mimic cartilage pathology in Kashin-Beck Disease (KBD)

The first media change consisted of removing both the insert and outside media from the culture system following a 48 hour incubation period followed by the addition of new media containing compounds to attempt to mimic changes seen in KBD.

The 24 well tissue culture plate was organised as shown in **Figure 2.4** to give three wells of each desired compound concentration.

A	**A**	**A**	**B**	**B**	**B**
C	**C**	**C**	**D**	**D**	**D**
E	**E**	**E**	**F**	**F**	**F**
G	**G**	**G**			

A Control

B Nivalenol 0.05µg/ml

C Nivalenol 0.1µg/ml

D Selenium 0.05µg/ml (Sodium selenite or Selenium-l-Methionine)

E Selenium 0.1µg/ml (Sodium selenite or Selenium-l-Methionine)

F Nivalenol and Selenium 0.05µg/ml

G Nivalenol and Selenium 0.1µg/ml

Figure 2.4 The layout of the 24 well cell culture plate

For the control group, DMEM containing 50µg/ml gentamycin and 20% heat inactivated FBS supplemented with 100µg/ml ascorbate and 5ng/ml TGFβ_2 was prepared.
The nivalenol and both selenium compounds were made up to 100µg/ml stock solution with DMEM and 20% heat inactivated FBS. This stock solution was then diluted with DMEM containing 50µg/ml gentamycin and 20% heat inactivated FBS to give concentrations of 0.1µg/ml and 0.05µg/ml and supplemented with 100µg/ml ascorbate and 5ng/ml TGFβ_2. Sufficient volumes of each were made up to enable thrice weekly media changes for a 4 week period, and in between each media change it was stored at -20^{o}C and subsequently thawed in a water bath.

Cultures were maintained by a media change thrice weekly; 600µl was pipetted to the outside well and 400µl into the inner insert. The cultures were maintained over a 4 week culture period at 37°C in a humidified atmosphere containing 5% CO_2.

Media was collected from the insert following each media change and stored at -20ºC until further analysis. Media from the outside well was discarded.

2.6 Harvesting scaffold free neo-cartilage grafts

Following a 4 week culture period the neo-cartilage grafts were harvested. This did not require an aseptic technique. The cartilage grafts were separated from the Millipore filter

insert membrane using a fine surgical forceps. The grafts were washed with PBS and blotted dry. The weight of the graft was then recorded and grafts for biochemical analysis were placed at -20ºC, grafts for histological analysis were fixed using 4% paraformaldehyde (4ºC) and grafts for immune- histochemical analysis were fixed using 95% ethanol until further analysis.

2.7 Cell Viability

The effect of Selenium (Sel-Met and Na-Sel) and/or Nivalenol on chondrocyte viability was determined using the MTT assay.

The MTT assay is a colorimetric assay used to measure the activity of enzymes that reduce MTT to formazan. The assay can be used to assess the viability and proliferation of cells and determine the cytotoxicity of agents/compounds.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. The insoluble intracellular formazan crystals are then dissolved using dimethyl sulfoxide (DMSO) into a purple coloured solution, the absorbance of which can then be measured using a spectrophotometer.

In order to establish the optimum cell density, different densities were tested and an MTT assay performed.

The method for the assay was;

- Chondrocytes were isolated from 7 day old bovine articular cartilage in the same manner as previously described.
- Cells were seeded as a monolayer at 0.5, 1, 1.5, 2, 2.5 or 3 x 10^6 cells/well within a 24 well in 1ml of serum free DMEM media containing gentamycin (50µg/ml) and 1% (v/v) HEPES buffer and allowed to adhere for 24 hours
- Media was removed and replenished and cells were cultured for a further 72 hours
- 200µl of media was removed and replaced with 200µl of 5mg/ml Thiazoyl blue tetrazolium bromide to give a volume of 1ml and cultured for 3 hours

- The 1ml was removed and the formed intracellular MTT crystals were dissolved by adding 200µl of DMSO
- 100µl from each well was transferred to a 96 well plate (see Figure 2.4)
- Absorbance was read at 540nm on a Labsystem Multiscan MS Spectrophotometer

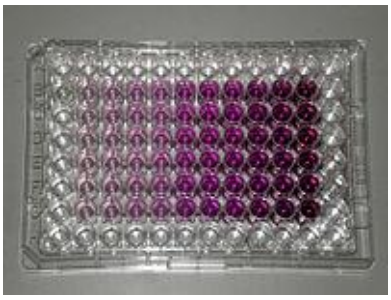


Figure 2.5 Photograph of a 96 well plate showing the purple formazan solution

As a result of this assay, it was decided that cells would be seeded at 0.5 x 10^6 to allow room for cell proliferation over the culture period in the subsequent experiments to test toxicity of Selenium and Nivalenol:-

- Chondrocytes were seeded within 24 well plates at 0.5 x 10^6 cells per well and allowed to adhere for 24 hours (a total of 8 plates were prepared)
- Media was removed and replaced with media containing no agents (Control), Selenium (Sel-Met or Na-Sel) and/or Nivalenol at concentrations of 0.05 or 0.1µg/ml
- Media was changed thrice weekly
- Cell viability (using the same method as described previously) was performed for day 7, 14, 21 and 28
- Absorbance was read at 540nm on a Labsystem Multiscan MS Spectrophotometer, with the results for Selenium and Nivalenol compared to that for the control

2.8 Histological analysis of scaffold free neo-cartilage grafts

For histology, one half of a pre-weighed cartilage graft for each treatment (the other half used for immune-histochemical analysis) was taken and fixed in cold 4% paraformaldehyde in PBS, and processed for paraffin wax using standard methods. Wax sections of the cartilage grafts (8μm in thickness) were cut using a microtome (LKB rotary microtome). Sections were de-waxed and dehydrated by putting them in descending alcohol concentrations as follows:

Xylene	2 min
Xylene	2 min
100% alcohol	1 min
100% alcohol	1 min
95% alcohol	1 min
70% alcohol	1 min

The sections were then washed in running water for 1 minute and then stained as follows:

1% (w/v) alcian blue pH 2.5	20 min
Wash in running water	1 min
Mayer's haematoxylin stain	1 min
Wash and blue in running tap water	5 min.
1% (v/v) eosin stain	2 min
Wash in running water	20 sec

The sections were then re-dehydrated and placed in an ascending alcohol concentration as follows:

70% alcohol	20 sec
95% alcohol	45 sec
100% alcohol	1 min
100% alcohol	2 min
Xylene	2 min
Xylene	2 min

The sections were mounted under a coverslip using DPX mountant and allowed to dry. Tissue organisation was evaluated using a brightfield microscope.

2.9 Immunofluorescent labelling of scaffold free neo-cartilage grafts

Circular 6mm tissue graft samples were harvested and stored at 4°C in a 95% ethanol solution. Prior to section cutting, individual tissue samples were put on rotation at 4°C in a PBS and 5% (w/v) sucrose solution (~5hours) to cryo-protect the grafts, then samples were snap frozen onto cryostat chucks in Cryo-M-Bed tissue mountant. Cryosections (12µm thickness) were cut using a Bright 5030 cryostat (Bright, UK). The sections were mounted onto HistoBond microscope slides and stored at -80°C until required. An area around each individual section was circumscribed with a water repellent ring using a DakoCytomation pen. The sections were then wet in 0.05M phosphate buffered saline (PBS) pH 7.4 containing 0.1% (v/v) Tween20 (Polyoxyethylene-Sorbitan Monolaurate) for 10 minutes. The sections were transferred to a humidified labelling box and pre-treated with appropriate enzymes to expose the neoepitope of interest or to improve antibody penetration (**Table 2.1**). This involved a pre-treatment with a cocktail of 0.4U/ml chondroitinase ABC and 0.4U/ml keratanase in a 100mM Tris Acetate solution (pH7.4) at 37°C for 1 hour to enzymatically de-glycosylate the sections. The sections were then washed in 0.05M PBS pH7.4 containing 0.1% (v/v) Tween20 (3x5 minutes). Blocking serum at 1:20 dilution was applied to the sections for 30 minutes at room temperature. Sections that were to be stained with monoclonal antibodies were blocked using goat serum and those to be stained with polyclonal antibodies were blocked with swine serum. The blocking serum was removed and primary antibodies applied at appropriate concentrations (**Table 2.1**) overnight at 4°C. As a negative control, the primary antibody was omitted and replaced with an appropriate (Mouse/Rabbit) 'naïve' immunoglobin (10µg/ml) with or without the enzymatic digestion step. Non-specific binding of the secondary antibody was checked by omitting the primary antibody and replacing it with 0.05M PBS and 0.1% (v/v) Tween 20.
Following an overnight incubation, the sections were washed in 0.05M PBS (pH7.4) containing 0.1% (v/v) Tween 20 (3x5 minutes). An appropriate FITC-conjugated secondary antibody (**Table 2.1**) recognising either mouse or rabbit species was applied at a 1:50 dilution for 1 hour at room temperature to individual sections. The sections were then washed in 0.05M PBS containing 0.1% (v/v) Tween 20 (3x5 minutes). Propidium Iodide (0.5µg/ml) was applied to sections for 5 minutes for nuclei staining, and then the sections were again washed in 0.05M

PBS containing 0.1% (v/v) Tween 20 (3x5 minutes). Sections were mounted under a coverslip (22x50mm) using vectashield fade retarding mountant, the coverslips were held into place using nail varnish. Sections were viewed and photographed using an Olympus fluorescence microscope.

Specificity	**Antibody** (clone; dilution)	**Pre-treatment**	**Blocking serum**	**Secondary antibody**	**Source/Reference**
Chondroitin-4-sulfate	2B6 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Couchman et al (1994) Caterson et al (1985)
Chondroitin-6-sulfate	3B3 (mono 1:20)	ABC/K	Goat	Goat Anti-mouse FITC	Caterson et al (1985)
Keratan sulfate	BKS1 (mono 1:20)	None	Goat	Goat Anti-mouse FITC	Caterson et al (1983)

Table 2.1 The required conditions for specific antibodies utilised in the immune-labelling of chief extracellular matrix molecules. ABC – chondroitinase ABC digested; K – keratanase digested.

2.10 Biochemical analysis of scaffold free neo-cartilage grafts- Proteoglycan extraction

Extraction of matrix molecules, including proteoglycans, present in neo-cartilage graft tissues was performed using guanidine HCl. The guanidine HCl extraction buffer was made up using the following concentrations;

- 4M Guanidine HCl
- 50mM sodium acetate
- 0.01M ethylenediaminetetraacetic acid disodium salt dehydrate
- 0.1M amino hexanoic (caproic) acid (pH 5.8-6.8).
- Fresh inhibitors were added prior to digest:

- 0.005M benzamidine HCl
- 0.5mM phenyl methyl sulfonyl fluoride (PMSF))

1ml of the solution was added to each graft for 48hours at 4°C with constant agitation. The 4M guanidine dissociates proteoglycan aggregates by breaking any non- covalent bonds and removing them from their native environment into solution (Sajdera and Hascall 1969), (Heinegard and Sommarin 1987). The mixture was centrifuged, the supernatant removed and dialysed three times against MilliQ™ water. The sulphated glycosaminoglycan content that was released by guanidine extraction was analysed using Dimethylmethylene Blue Assay (DMMB) and the collagen concentration was analysed via a hydroxyproline assay. The insoluble grafts or residue were then digested with pepsin for further analysis.

2.11 Pepsin digest

Following a guanidine extraction the residual grafts were digested using pepsin with the aim of releasing soluble type II collagen. Pepsin is an endopeptidase that hydrolyses peptide bonds.

Each sample was placed in 0.5ml of 0.5M acetic acid containing 1mg/ml pepsin for a period of 24hours at 4°C with constant agitation. The sulphated glycosaminoglycan content that was released by the pepsin digest was analysed using Dimethylmethylene Blue Assay (DMMB) and the collagen concentration was analysed via a hydroxyproline assay. The insoluble grafts or residue were then digested with papain for further analysis.

2.12 Papain digest

The residual grafts still remaining were completely digested via a papain digest. Papain is an extremely stable cysteine protease that works at elevated temperatures. It digests most protein substrates more extensively in comparison to other proteases. The addition of 1ml papain buffer (*0.05M sodium-acetate, 0.025M EDTA pH 5.6, 0.005M cysteine and 2µl/ml papain*) was added to each graft for 18hours at 65°C with occasional agitation. The sulphated glycosaminoglycan content that was released by papain extraction was analysed using Dimethylmethylene Blue Assay (DMMB) and the remaining collagen concentration was analysed via a hydroxyproline assay.

2.13 Analysing sulphated GAG concentrations using Dimethylmethylene Blue Assay (DMMB)

Both media and graft proteoglycan content was measured as sulphated GAG using Dimethylmethylene Blue (DMMB) assay. DMMB binds to the negatively charged sulphate groups on the GAG chains forming a complex which produces a metachromatic shift in the absorbance maxima (Farndale, Buttle et al. 1986).

Standards ranging from 0-40μg/ml GAG were prepared using chondroitin sulphate C from shark cartilage. The assay was performed by adding 40μl of these standards in triplicate, as well as appropriately diluted samples (with the dilution factor used to calculate final GAG concentration), to a 96 well multi-plate. 200μl of DMMB solution (*32mg 1,9 DMMB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98% formic acid and made up to 2L with water*) was added to samples and absorbance was read at 525nm on a Labsystem Multiscan MS Spectrophotometer.

A mean for each sample was taken. This was also performed for the standards to ensure accuracy of the data.

2.14 Media analysis for lactate concentrations

The lactate assay was carried out using the Trinity biotech assay kit (Procedure No 735).

Lactate is a by-product of the glycolytic pathway and is utilised as a measure to determine the anaerobic metabolic activity that occurs in a culture system. Lactic acid is converted to pyruvate and hydrogen peroxide by lactate oxidase. In the presence of the hydrogen peroxide formed, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye, with an absorption maximum at 540nm. The increase in absorbance at 540nm is directly proportional to lactate concentration.

Lactate standards of 0μg/ml, 25μg/ml, 50μg/ml, 100μg/ml, 200μg/ml, 300μg/ml and 400μg/ml were added (5μl) in duplicate to a 96 multi-well plate. Culture media for each sample, (also 5μl at appropriate dilutions) was added in triplicate. To each well 250μl of

lactate reagent (*lactate oxidase (microbial), peroxidase (horseradish), chromogen precursors and buffer pH 7.2*) was pipetted and then the multi-well plate was incubated at room temperature for 10 minutes. The absorbance at 540nm was read using the Labsystem Multiscan MS Spectrophotometer.

A mean for each sample was taken. This was also performed for the standards to ensure accuracy of the data.

2.15 Hydroxyproline Assay

Hydroxyproline is unique to collagen. As a result it is used to determine to quantity (but not types) of collagenous protein present in tissue or a culture medium (Woessner 1961), (Reddy and Enwemeka 1996). The hydroxproline content generated by the Transwell culture system was analysed, determining the amount of collagen retained within grafts (following a Guanidine extraction, Pepsin digest and Papain digest) and within the media.

Samples were initially hydrolysed in 6M HCl for 24 hours at 110ºC (this generates free hydroxyproline not peptide bound for analysis). Hydroxyproline is fairly stable in 6M HCl although hydrolysis does initiate the epimerization of hydroxyproline, but in this colorimetric assay all epimers are determined together. Following the hydrolysis process the hydrolysates are freeze dried to remove the acid. The freeze dried samples are then reconstituted with milli Q water in the same volume of media that was originally taken for hydrolysis. The reconstituted sample may contain a lot of particulate materials, therefore samples were centrifuged at 8000rpm for 10 minutes in a microfuge to sediment insoluble material that could interfere with the assay.

In a combi-plate 8 ELISA plate, trans–4–Hydroxy–L-proline standards; 30µl of 0µg/ml, 0.5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, and 10µg/ml were added in triplicate and 30µl of sample at appropriate dilutions were also added in triplicate. To each well 70µl diluent (*100ml propan-2-ol and 50ml water)* and 50µl oxidant (*0.308M Chloramine T, 10ml water and 50ml stock buffer (Stock buffer; 0.42M sodium acetate trihydrate, 0.127M tri sodium citrate dehydrate, 0.025M Citric acid and 40% (v/v) Propan-2-*

ol)) was added and the plate was mixed on a plate shaker for 5 minutes. The oxidant was prepared fresh prior to use.

The addition of the oxidant oxidises the water soluble hydroxyproline to the toluene soluble pyrrole molecule, this chemical conversion is advantageous as small amounts of hydroxproline can be detected in samples containing large amounts of contaminating substances. Colour reagent (again prepared fresh prior to use; *0.682M ρ-dimethylaminobenzaldehyde (Ehrlich's reagent) 11.25ml (60% V/V) perchloric acid and 62.5ml propan-2-ol)* was added to the plate at 125µl. The plate was then mixed well on a plate shaker and incubated at 70ºC for 20 minutes. The addition of *ρ*-dimethylaminobenzaldehyde reacts with the pyrrole molecules that were generated by oxidation and forms a complex that is detected and quantified as a chromophore (peach colour) at 550nm. A graph was plotted of absorbance versus concentration of standards and a line of best fit plotted (using Microsoft Excel) giving an equation of the function for the straight line. The equation was then used to calculate the concentration of hydroxyproline present in the samples. The content of hydroxylated proline residue was used to estimate collagen content of the cartilage.

2.16 Size Exclusion Column Chromatography

Size exclusion chromatography is a method of separating molecules within a solution according their size and/or molecular weight. The underlying principle is that particles of different sizes will elute (filter) through a stationary phase at different rates. The column consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. These pores may be depressions on the surface or channels through the bead.

The solution to be separated passes down the column, with the smaller molecules entering the pores, but the larger ones, which cannot enter into as many pores pass through the column. The filtered solution collected at the end of the column is called the eluate. The larger the molecule is, the faster the elution and therefore the small molecules that can enter all of the pores will elute late. The void volume includes any particles too large to enter the medium, and the solvent volume is known as the column volume.

Sepharose CL-2B was obtained from Amersham Biosciences. Sepharose is a brand name derived from **Se**paration-**Pha**rmacia-Aga**rose**. It is a cross-linked, beaded form of a polysaccharide polymer material extracted from seaweed. It is cross-linked by lysine side chains. The sepharose CL-2B was placed into a 110cm x 2.5cm glass column and allowed to settle. The column was washed through with a buffer of 50mM sodium acetate (pH 6.8) before being used each time. In between uses it was preserved with 20% ethanol solution to prevent 'drying out'.

The column was calibrated using the AKTAexplorer automated liquid chromatography system. An elution buffer of 50mM sodium acetate and 100mM sodium sulphate (pH 6.8) was used and 4ml fractions were collected at a rate of 1ml per minute. These fractions were then assessed for sGAG using the DMMB assay as previously described, and results were plotted on a graph.

In order to establish standards for aggregated aggrecan and monomer, aggregated aggrecan (A1) and monomer (A1D1) samples from bovine nasal cartilage were used, with 500µg of sGAG loaded and results plotted on graphs.

For media, samples from weeks 1 and 2 (one ml from each) were pooled, and for weeks 3and 4. The quantity of sGAG in these samples was already established from previous DMMB assays. In those samples with low sGAG content, a larger volume was pooled and dried on a speed vac to give a 2ml volume to be loaded on the column. Fractions collected were analysed using the DMMB assay and results plotted for comparison to the standards. Some samples contained such little sGAG that results were deemed unreliable.

For analysis of the cartilage graft, the 4M guanidine extraction samples were used after being dialysed against MilliQ H_2O. For those samples with a known high sGAG content 1ml was loaded, intermediate content 2ml was loaded and for low content samples were pooled and dried to a 2ml volume using a speed vac. Fractions collected were analysed using the DMMB assay and results plotted for comparison to the standards. Similarly to the media, some samples contained such little sGAG that results were deemed unreliable.

2.17 Statistical Analysis

Results for mean, standard deviation and standard error were calculated using standard calculations.

Comparative statistical analysis was carried out using the Kolmogorov-Smirnov Test. This was chosen over the Students t-test as it makes no assumption about the distribution of data.

CHAPTER 3

DEVELOPMENT OF AN *IN VITRO* CULTURE SYSTEM THAT MIMICS CARTILAGE PATHOLOGY IN KASHIN-BECK DISEASE

3.1 Preliminary studies

The aim of the studies described in this chapter was to try and establish appropriate doses of Selenium (in this case Sodium selenite; Na-Sel) and Nivalenol (NIV) to add to an *in vitro* Transwell cartilage culture system in an attempt to mimic metabolic changes seen in cartilage metabolism in KBD.

The cartilage culture system was the same as has been described in the methods (section 2.2), except that the concentrations of Na-Sel and NIV used were 0.1, 0.5 and 1µg/ml. Harvesting of the graft (and subsequent weighing) and collection of the media were performed in the same manner. The graft was extracted with 4M Guanidine HCl and the residue then digested with papain. The sGAG content of the media, extract and residue were assessed with the DMMB assay. Media was also assessed with a lactate analysis as a measure of biochemical cell viability. In addition, selected cartilage grafts for each treatment were divided into 2 equal parts, with one part prepared for histological assessment and the other for immunohistochemistry. A summary of these methods are shown in Figure 3.1.

3.2 Preliminary study Results:

Histological analyses (using Alcian Blue staining) of the neocartilage grafts grown in Transwell cultures in normal culture media (Control), 0.1 & 0.5µg/ml Sodium Selenite (Na-Sel) or 0.1 & 0.5µg/ml Nivalenol (NIV) are shown in Figure 3.2(a). These histology sections show that the grafts grown under normal culture conditions produce large neocartilage grafts with zonal stratification similar to that seen in native hyaline articular cartilage (Hayes et al, 2007). In the presence of 0.1µg/ml of sodium selenite this ‘native’ zonal organisation is still maintained although the graft is somewhat smaller. At 0.5µg/ml sodium selenite the graft is

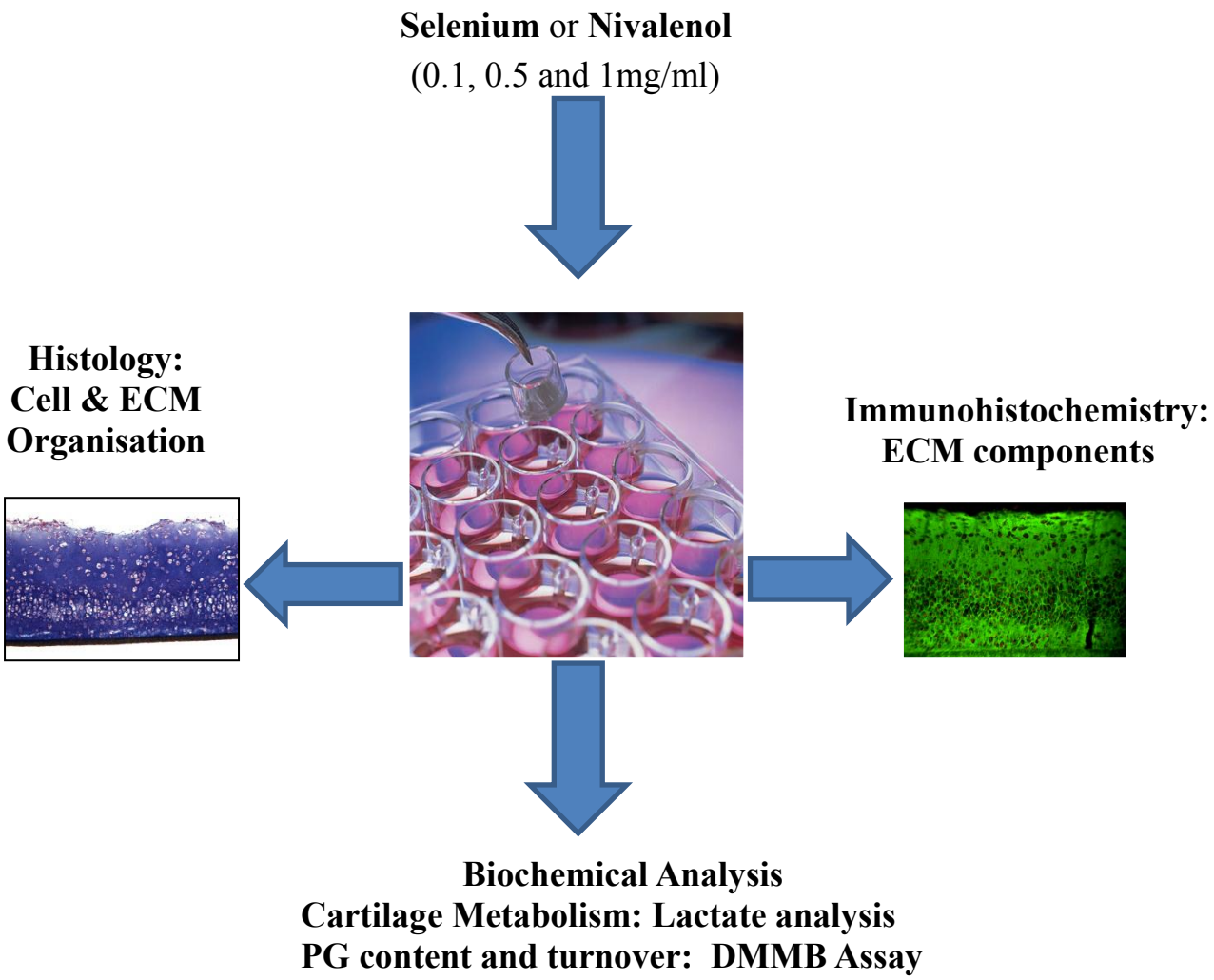


Figure 3.1 Schematic review of procedures used

considerably smaller with less Alcian Blue staining (indicative of less proteoglycans present) and with loss of the zonal organisation seen in native articular cartilage. Neocartilage explants grown in the presence of 0.1 or 0.5µg/ml Nivalenol showed very diminished Alcian Blue staining indicative of there being considerably less proteoglycan present in these neocartilage explants. The size of the neocartilage explants were also very much smaller than the Controls or those grown in the presence of sodium selenite. The reduction in proteoglycans present in the grafts grown in the presence of Nivalenol would also contribute to their reduced size because of these grafts having a reduced capacity for water retention a property endowed by the proteoglycans present in normal cartilage. The wet weights of the

different neocartilage grafts developed in the different culture conditions were also determined (Figure 3.2(b)).

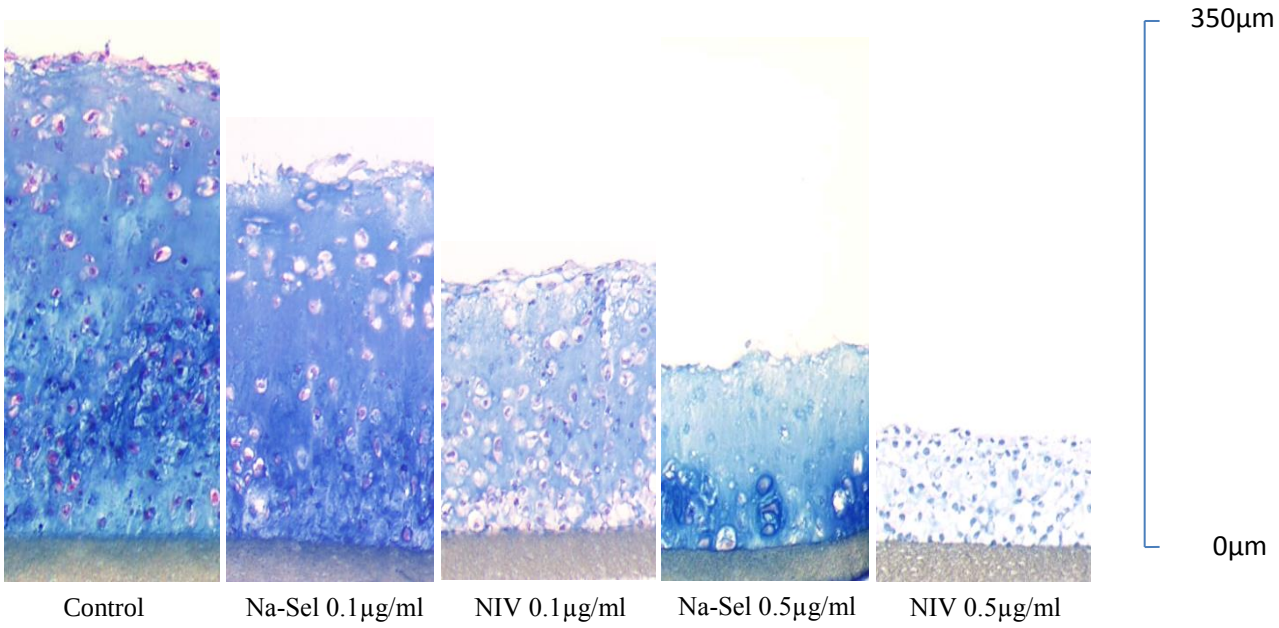


Figure 3.2(a) Histology slides following Alcian Blue staining of grafts following harvest for selected treatments

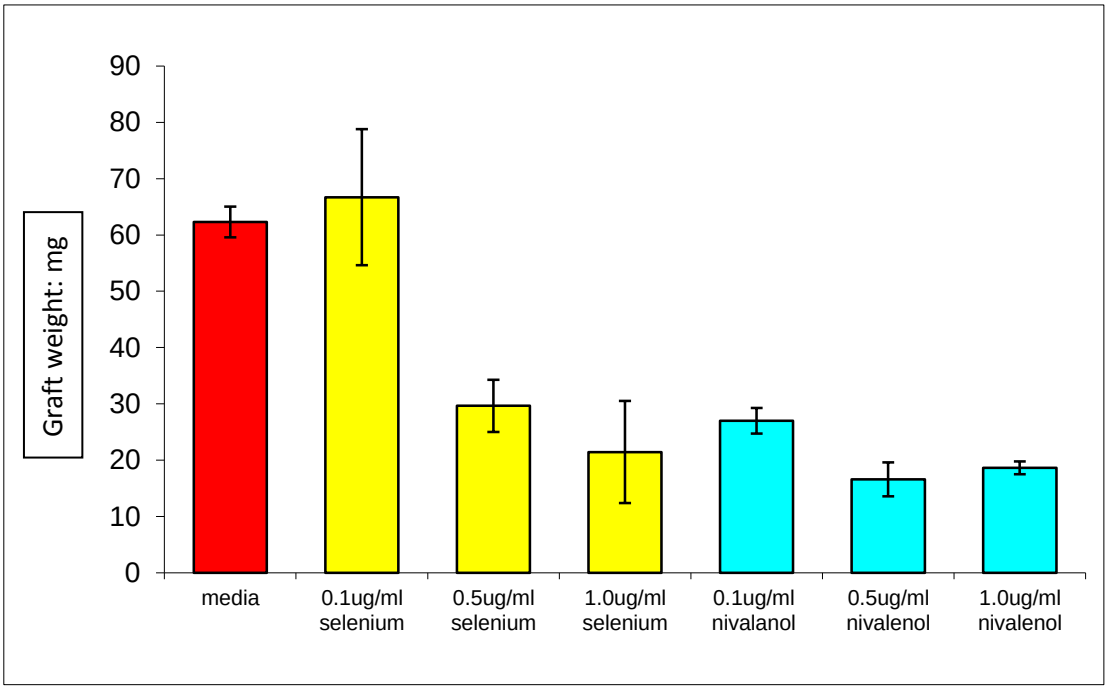


Figure 3.2(b) Graph showing graft wet weight (mg) for each treatment (n=3)

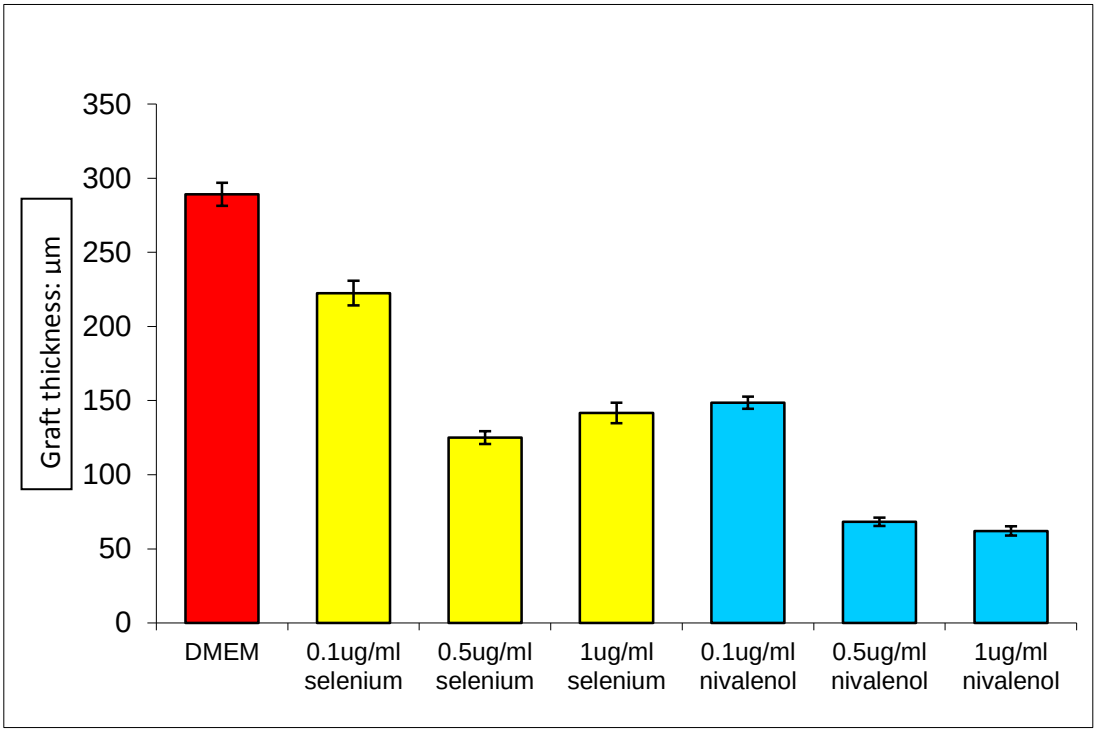


Figure 3.2(c) Graph showing graft thickness (µm) for each treatment (n=3)

The wet weights of the cartilage neografts grown in Control media and 0.5µg/ml sodium selenite were essentially the same. However, those grown in media with higher concentrations of sodium selenite (0.5 & 1.0 µg/ml) and all concentrations of Nivalenol were significantly lower weights than the Control these differences likely reflecting the reduction in their proteoglycan content as described above. Measurements of graft thickness, seen in Figure 3.2(c), were similar to the weight measurements except that the cultures grown in the presence of 0.1µg/ml sodium selenite were less than that found in the control.

The viability of the cells present in the neocartilage grafts grown under the different culture conditions was also investigated (Figure 3.3(a)). Here, the lactate concentration (a measure of cellular metabolism) in the media of cartilage neografts for 1, 2, 3 & 4 weeks was highest in the control cultures and slightly lower in cultures grown in the presence of 0.1µg/ml sodium selenite. However, in the presence of higher amounts of sodium selenite and all concentrations tested for Nivalenol the lactate levels were much lower than the controls, this lessening of the lactate content possibly due to lower cell numbers present in the cartilage neografts. The lowest lactate levels were seen in the presence of 1.0 µg/ml Nivalenol in the culture medium.

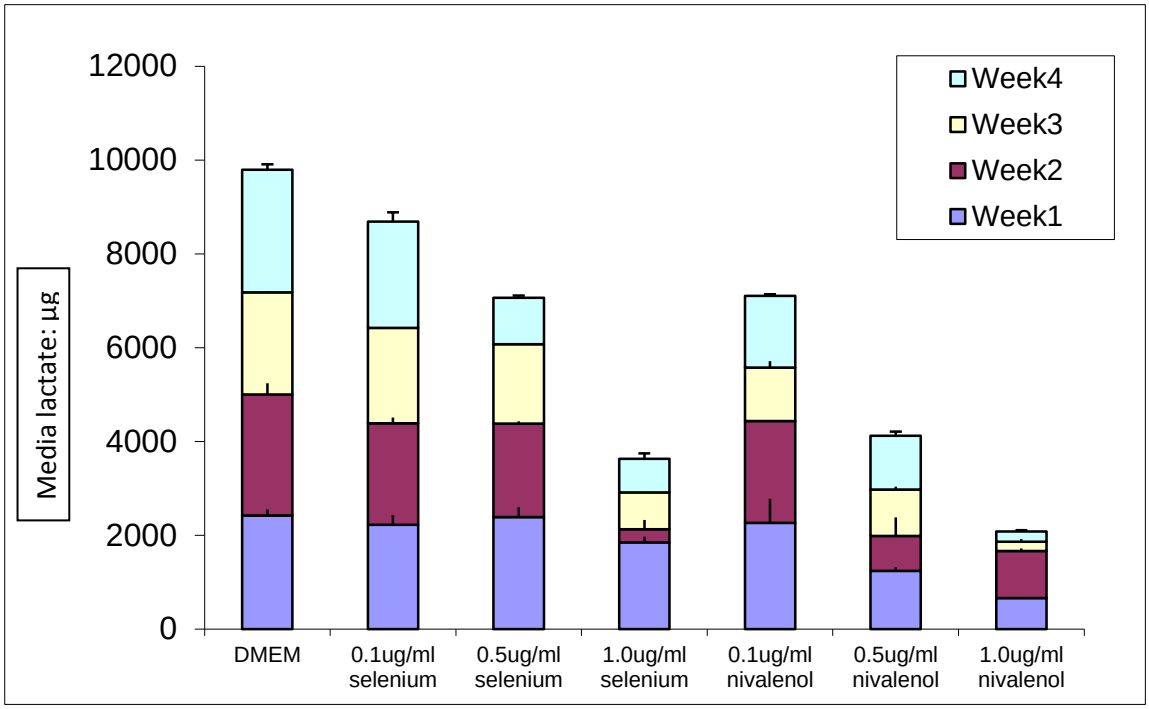


Figure 3.3(a) Graph showing media lactate (µg) per week for each treatment (n=3)

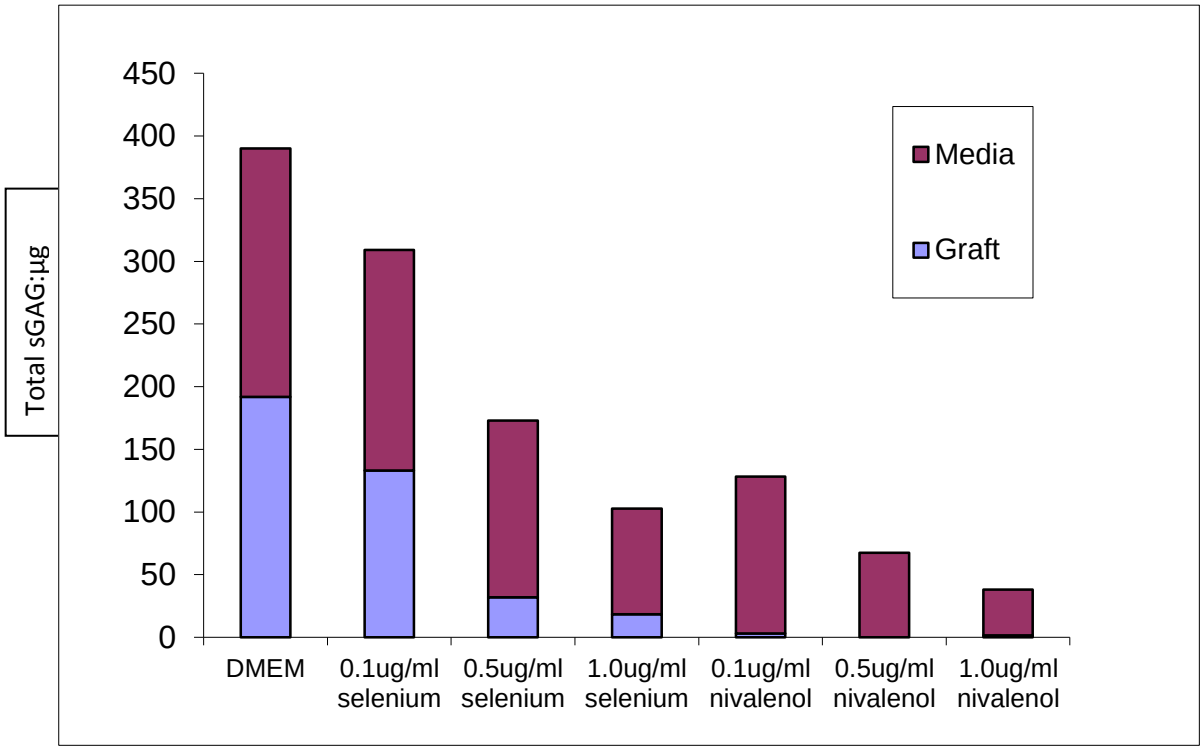


Figure 3.3(b) Graph showing sGAG (µg) content in the media and graft per week for each treatment (n=3)

Figure 3.3(b) shows the sGAG content of the Transwell culture media and 4M guanidine HCl extracts of the cartilage neografts grown either in Control media or in the presence of sodium selenite or Nivalenol. These results show that the greatest production of sGAG (proteoglycans) occurred in the Control media where there was an approximate 50:50 split of the sGAG present in each compartment; i.e. the media and the neograft cartilage. However, the presence of sodium selenite in the media caused a reduction in the total sGAG present as well as a 60:40 per cent distribution between the culture media and the cartilage neograft. The presence of higher contents of sodium selenite (0.5 &1.0 μg/ml) and all concentrations of Nivalenol in the media caused a very significant drop in the total sGAG produced as well as a disproportionate proportion of the sGAG being present in the media with very little present in the cartilage neograft itself.

Immunohistochemical data shown in Figure 3.4 indicates that the major chondroitin sulphate sulphation isomer synthesised on the matrix proteoglycans present in the model Transwell culture system is chondroitin-4-sulphate; i.e. there is no detectible 3-B-3(+) positive 6-sulphated "CS stubs" detected in the cartilage neografts. In contrast, strong staining for 2-B-6 (i.e. 4- sulphated "CS stubs") were seen in all cultures (Figure 3.4 D, E & F). However, this staining was significantly reduced in cultures grown in the presence of 0.1μg/ml sodium selenite or Nivalenol, data that is consistent with the sGAG analyses of the grafts shown in Figure 3.3. However, the occurrence of the bigger differences between the 0.1μg/ml Nivalenol (almost 0 in Figure 3.3) and 0.1μg/ml sodium selenite are not as apparent in Figure 3.4. It is also interesting to note that whilst the C-4-S (2-B-6 positive) staining is fairly even throughout the depth of the Control cartilage grafts (Figure 3.4 D) the strongest 2-B-6 positive staining was found in the deeper zones of the 0.1μg/ml sodium selenite and Nivalenol grafts. Interestingly, keratan sulphate GAG staining (detected with mAb BKS-1 after keratanase treatment) was fairly evenly distributed throughout the Control grafts (Figure 3.4 G) with more of it present in the surface zones for both the Control and 0.1μg/ml sodium selenite grafts (Figure 3.4 H) but there was more staining present in the deeper zones of the 0.1μg/ml Nivalenol exposed grafts (Figure 3.4 I). These immunohistochemical analyses are consistent with the sGAG patterns shown in Figure 3.3.

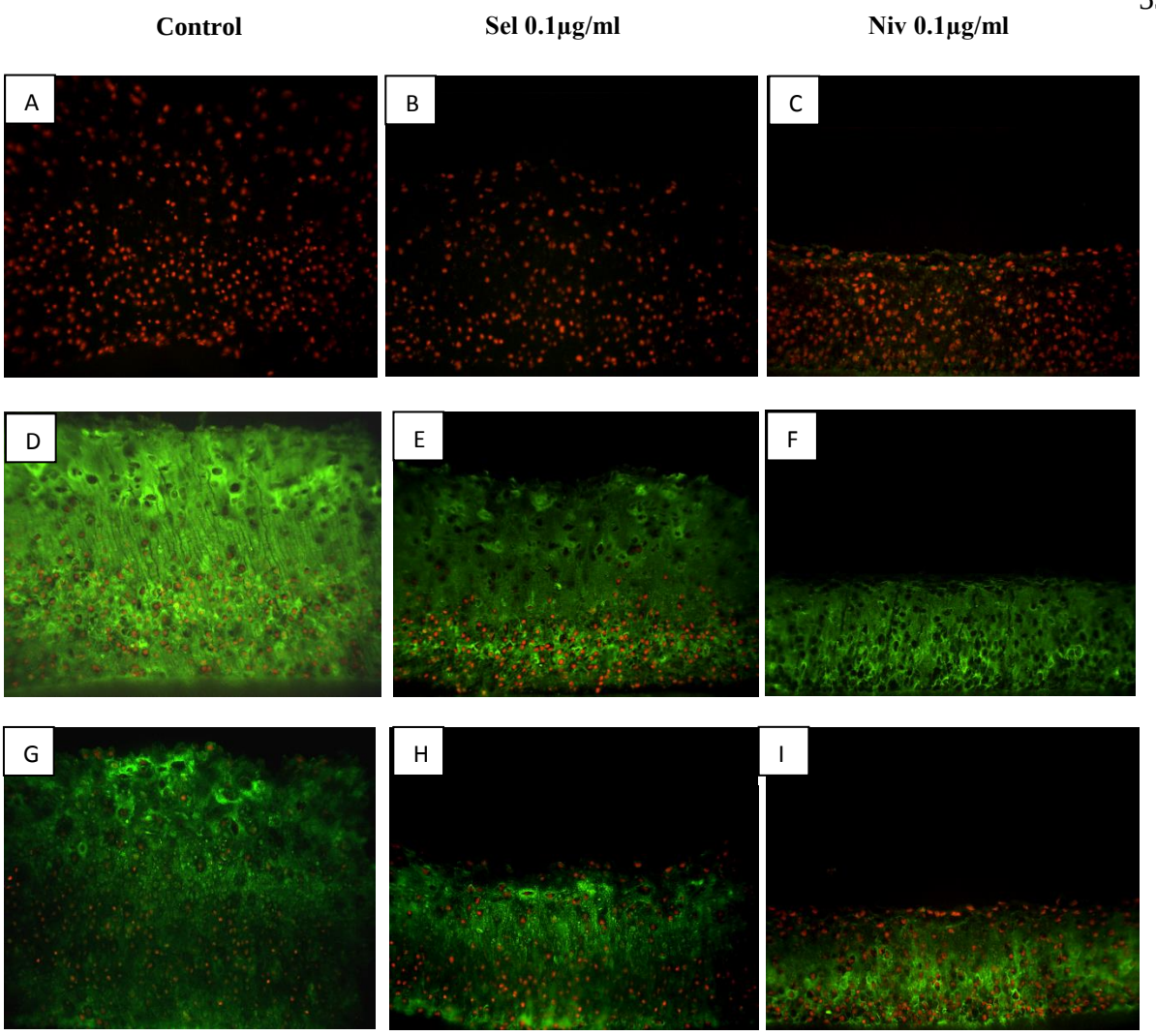


Figure 3.4 (a-i) Immuno- histochemistry comparing results for Control, Selenium (0.1ug/ml) and Nivalenol (0.1ug/ml) staining for C-6-S using 3B3 + ABC (a-c), C-4-S using 2B6 + ABC (d-f) and KS using BKS1 +K (g-i)

3.3 Introduction: Determination of the effects on cartilage neograft formation of different sources of selenium either alone or in combination with Nivalenol exposure

Kashin-Beck disease (KBD) is an endemic osteoarthropathy with pathological changes occurring in the growth plate and articular cartilage in humans. It is postulated that KBD is due to the presence of fungal mycotoxins that infiltrate the diet, and a regional selenium deficiency in the environment providing local food sources in a broad belt across China. There is limited understanding of the condition with only small amounts of research

being carried out on this disease. *In vitro* studies that have investigated the main "hypothesised" causes for the development of KBD (presence of mycotoxins and deficiency of selenium in the diet) have concentrated on the use of chondrocyte monolayer cultures. Although the use of these culture systems can yield important information about the metabolic state of the chondrocytes and the production of an extracellular matrix, none of these studies have examined the effect on cartilage graft development *per se*. Thus, the objective of this study was to determine the effects different sources of selenium, in the presence or absence of Nivalenol, had on cartilage neograft metabolism.

The first consideration in the 'native' development of chondrocyte cultures that mimics the development of native hyaline cartilage is to establish the correct culture conditions. Articular cartilage is a relatively simple tissue with a low cell-to-matrix volume containing a single cell type; the chondrocyte, and it has no innervation, blood or lymphatic supply (Poole, Kojima et al. 2001). The mature state of articular cartilage consists of four distinct zones; superficial, middle, deep and a zone of calcified cartilage. Within every zone the morphology, gene expression, biochemical composition, and collagen fibre organisation of the chondrocyte differs. Together, these characteristics allow articular cartilage to resist and dissipate biomechanical load while providing smooth, low friction joint articulation for movement.

Previous studies, described earlier in this chapter, have shown that neo-cartilage grafts produced *in vitro* from a high density, immature, primary chondrogenic source using a Transwell culture system, had the ability to reconstitute a highly organised stratified hyaline tissue showing similarities to native adult articular cartilage following just 2 weeks in culture (Kandel et al, 1995). Studies within our group have added to the data generated from Kandel et al, 1995, and demonstrated that chondrocytes grown at high density upon porous filter membranes can produce zonally stratified neo-cartilage grafts for potential use in the biological repair of intra-chondral lessons of articular cartilage (Hayes, Hall et al. 2007). All tissues engineered from a primary chondrocyte source within this study showed histological and ultra-structural similarities to native articular cartilage. These neo-cartilage tissues comprised three histological distinct zones that resembled the superficial, middle and deep zones of native articular cartilage, and within these zones there were striking similarities in cell morphology, collagen fibre organisation, and matrix composition to that of mature articular cartilage (Hayes, Hall et al. 2007). Therefore, this Transwell culture system is an

excellent model to validate an *in vitro* culture system that mimics cartilage pathology in KBD.

The primary aim of the research in this experimental chapter was to use this Transwell culture system described above to establish a culture system model to mimic articular cartilage pathology in KBD *ex vivo*.

3.4 Optimisation of cell seeding concentrations to assess viability

The effect of sodium selenite (Na-Sel) or selenium-1-methionine (Sel-Met) and/or Nivalenol (NIV) on chondrocyte viability was determined using the MTT assay which measures uptake and subsequent release (following cell lysis) of MTT formazan (section 2.3). Firstly, the optimum cell density was determined. Bovine chondrocytes were isolated from 7 day old bovine articular cartilage following collagen/pronase digestion and seeded at 0.5, 1, 1.5, 2, 2.5 or 3 x 10^6 cells/well within a 24 well plates (as a monolayer), and allowed to adhere for 24 hours. The media was then removed and replenished. The cells were cultured for a further 72 hours at which point the MTT assay was performed and the intensity of the MTT conversion was measured at 540nm.

Figure 3.5 illustrates the relationship between MTT assay absorbance and cell number. The relationship between absorbance and cell number is not linear. Wells containing 0.5x10^6 and 1x10^6 cells showed a similar absorbance. However, cells seeded 1.5 x10^6 and beyond demonstrated a decline in absorbance suggesting cellular saturation, and as a possible consequence cell death. Therefore, for the subsequent experiment to determine the effect of SEL and/or NIV on cellular viability, the cells were seeded at 0.5 x 10^6 to allow room for cell proliferation over the culture period.

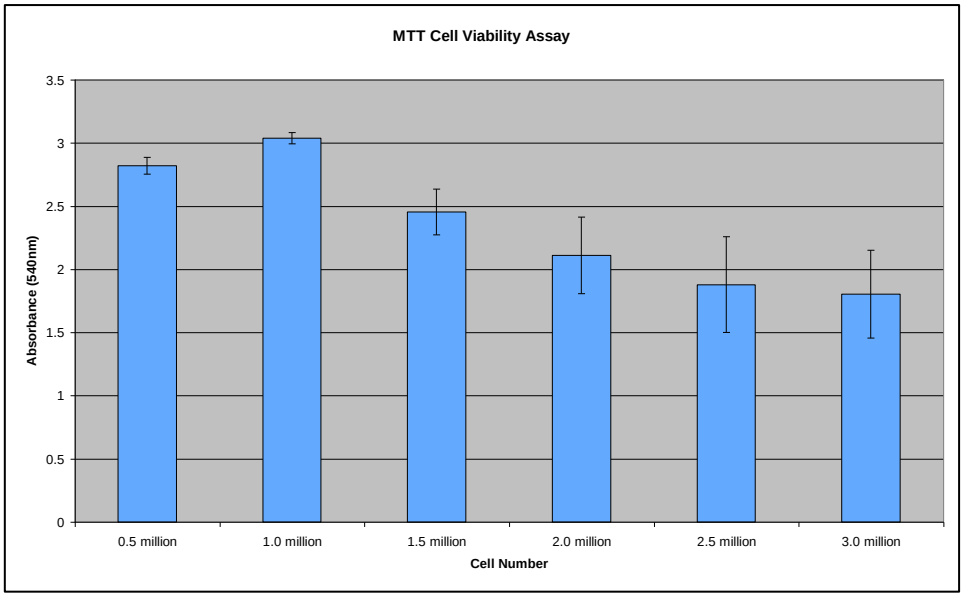


Figure 3.5 Determining the optimal cellular number (MTT). (n=3)

3.5 Effect of selenium (SEL), Nivalenol (NIV) or in combination on bovine chondrocytes viability *in vitro:* MTT Assay

To test the effects of selenium (either sodium selenite or selenium-1-methionine) and Nivalenol, cells were seeded in 24 well plates at 0.5 x 10^6 cells per well as a monolayer and allowed to adhere for 24h after which media was removed and replenished. Media either contained no added agents (Control), or contained selenium-l-methionine (Sel-Meth) or sodium selenite (Na-Sel) and/or Nivalenol (NIV) at concentrations of 0, 0.05 or 0.1µg/ml over a 4 week period. Media was changed thrice weekly and cell viability using the MTT assay was examined on day 7, 14, 21 & 28.

Figure 3.6 shows that at days 7 and 14 there was no significant changes between any of the culture conditions for cellular viability maintained under these culture conditions. However, at days 21 and 28 there was notable decline in cell viability in Transwell cultures grown in the presence of NIV supplemented cultures when compared to the Control. However, in the presence of both selenium additives (i.e. Sel-Meth & Na-Sel at both 0.05 and 0.1µg/ml) the cell viability in the presence of NIV showed a recovery at both time points; day 21 & day 28. These results suggest that the presence of selenium can counteract the bad cell viability effects of NIV on the chondrocytes in these Transwell culture systems.

3.6 Lactate Assay for cellular metabolism

Cells were seeded within 24 well Transwell cultures at 4 x 10^6 cells/well allowed to adhere for 24h after which the media was removed and replenished containing no compounds (Control), Na-Sel or Sel-Meth and/or NIV at concentrations of 0.05 or 0.1µg/ml over a 4 week period. Media was changed, and harvested thrice weekly. The media harvested was pooled together for each week (i.e. Week 1 to 4). The media was analysed using a lactate assay. Figure 3.7 shows fairly consistent results in each experiment (a & b), with lactate levels differing very little between treatments.

3.7 Effect of selenium (SEL), Nivalenol (NIV) or both in combination on cartilage graft weight

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion and seeded at 4 x 10^6 cells into Millipore filter Transwells within 24 well plates. Media was changed thrice weekly and cultured for 28 days. At the termination of culture the media from the Transwell was removed/ harvested and the resulting cartilage graft was weighed.

Figures 3.8 (a) shows the results of graft wet weights for each culture condition in the experiment using no additives (Control), Nivalenol alone, sodium selenite or selenium-1-methionine alone or in combination with Nivalenol. The cartilage grafts grown in the presence of NIV alone significantly decreased in a dose dependent manner compared to the Control ($p<0.05$). The graft weight was significantly more when cultured in the presence of 0.05µg/ml Na-Sel and NIV or 0.1µg/ml Na-Sel and NIV when compared to 0.05µg/ml or 0.1µg NIV alone ($p<0.05$). There were no significant differences between the 2 concentrations of Na-Sel alone compared to the control ($p>0.05$).

Figure 3.8 (b) shows the graft wet weights for each culture condition in the experiment using selenium-l-methionine. The cartilage graft grown in the presence of NIV at 0.1µg/ml was significantly decreased to the Control ($p<0.05$), but at 0.05µg/ml NIV the difference was not significant. The combination of both Sel-Met and NIV at both doses produced a cartilage graft with a significantly lower weight than the control. There were no significant differences between the 2 concentrations of Selenium-1-methionine alone compared to the control ($p>0.05$).

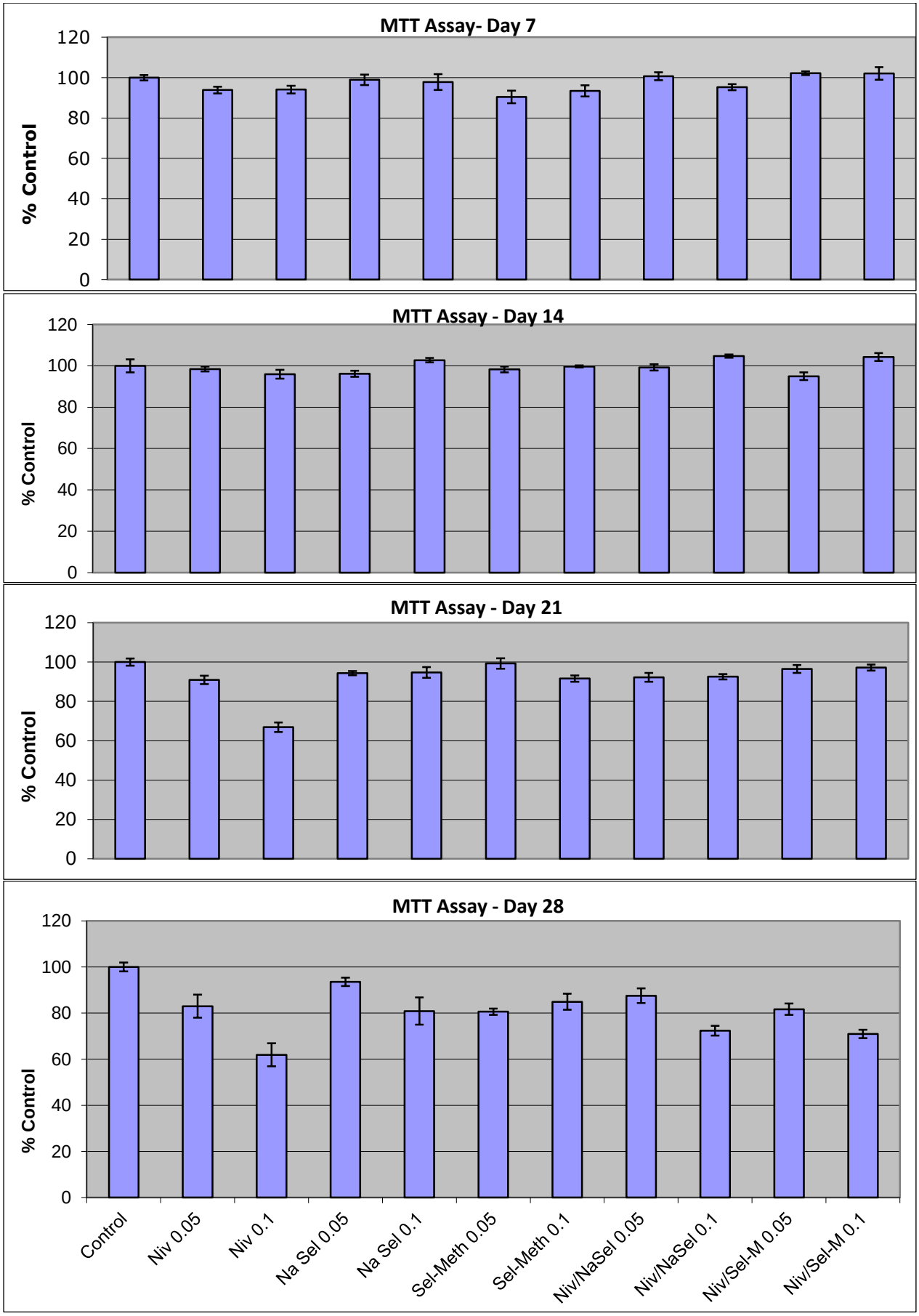
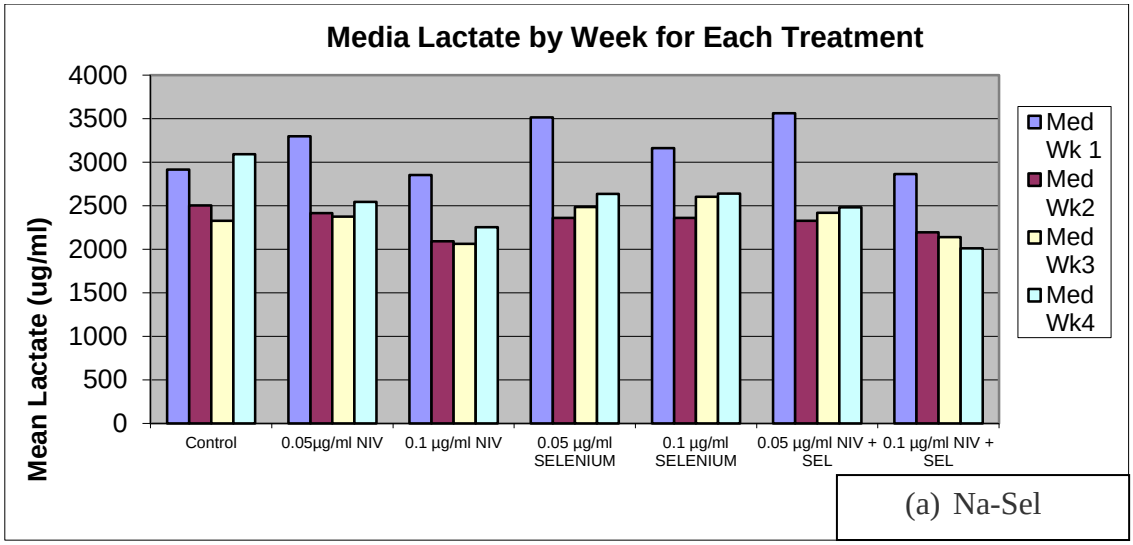


Figure 3.6 The effect of two different selenium additives (Na-Sel & Sel-Meth), Nivalenol (NIV) separately or in combination on bovine chondrocytes cell viability (MTT assay) in cartilage grafts from Transwell in vitro cultures maintained for 7, 14, 21 & 28 days. (n=6)



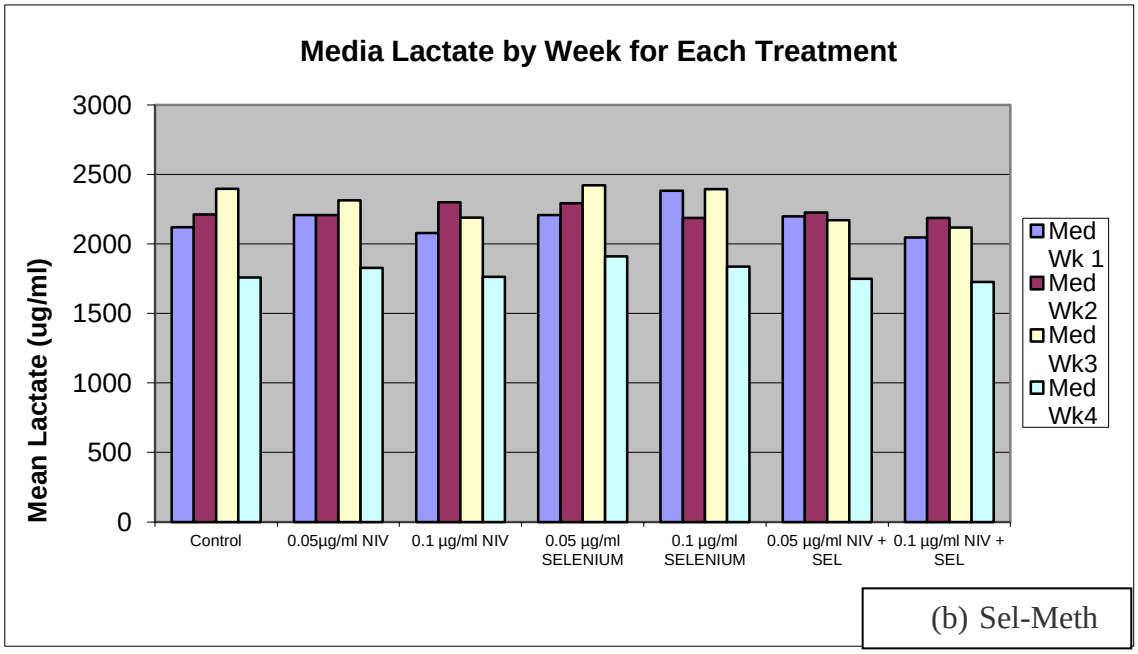


Figure 3.7 Effect of selenium ((a) Sodium selenite, (b) Selenium-l-methionine), Nivalenol or in combination on lactate released into the culture media (n=9)

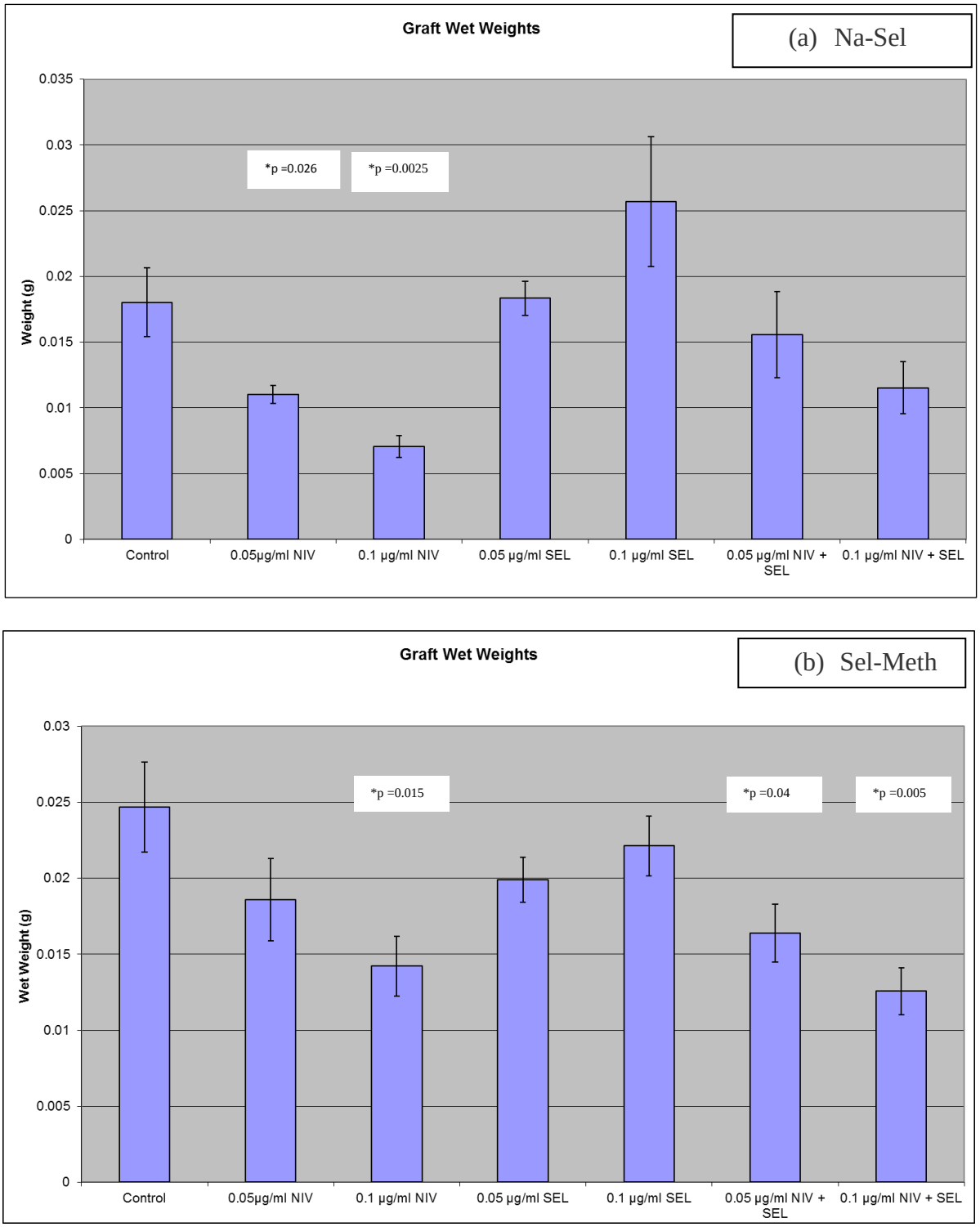


Fig 3.8 Effect of Selenium (SEL), either (a) sodium selenite, (b) selenium-l-methionine with or without Nivalenol (NIV) on neocartilage tissue graft wet weight (n=9)

3.8 Histological analysis of neocartilage grafts grown in the presence of sodium selenite (Na-Sel), Nivalenol (NIV) or in combination.

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly and cultured for 28 days. At the termination of culture the grafts were fixed in 2% paraformaldehyde and processed into paraffin wax, sectioned and mounted on slides. Sections were then stained for proteoglycan content with 1% (w/v) Alcian blue and H&E.

Figure 3.9 shows the histology staining for each culture condition. Grafts cultured in the absence of either NIV or SEL (Fig 3.9a Control) demonstrates the three histologically distinct zones that correlate to the surface, middle and deeper zone of native hyaline articular cartilage. The surface zone was weakly alcianophilic consisting of discoidal cells. In this zone, the cells appeared to have a prominent horizontal organisation. The under-lying mid-zone was more alcianophilic, indicating a higher proteoglycan content than the surface zone, and contained spheroidal cells that are separated by large amounts of extracellular matrix. The subjacent deep zone was more cellular in appearance than the above cartilage zones.

Cells in this zone were ovoid, hypertrophic in morphology and densely packed together and highly alcianophilic indicating high content of proteoglycans. In comparison to control, in the presence of 0.05µg/ml NIV (Fig 3.9b) the arrangement of the 3 cartilage zones are less defined. The surface zone in the presence of 0.05µg/ml NIV still contains cells that appear discoidal in appearance but have a less noticeable horizontal organisation. The middle zone was still alcianophilic in the presence of 0.05µg/ml compared to the surface layer indicating an increase presence of proteoglycans. The organisation of the cells within the middle layer appears to have a more scattered appearance compared to control and merge with the deep zone. The deep zone contains increased staining compared to the upper adjacent layer but to a less degree in the presence of 0.05µg/ml compared to the control. In the presence of 0.1µg/ml NIV (Fig 3.9c), the organisation of the cartilage zones is even more disrupted compared to the control. There is still a defined surface layer with horizontal, discoidal cells with weak staining. However, there is no distinct middle or deeper zone layers with it being much less alcianophilic compared to the Control, and contains a mixture of cellular shapes.

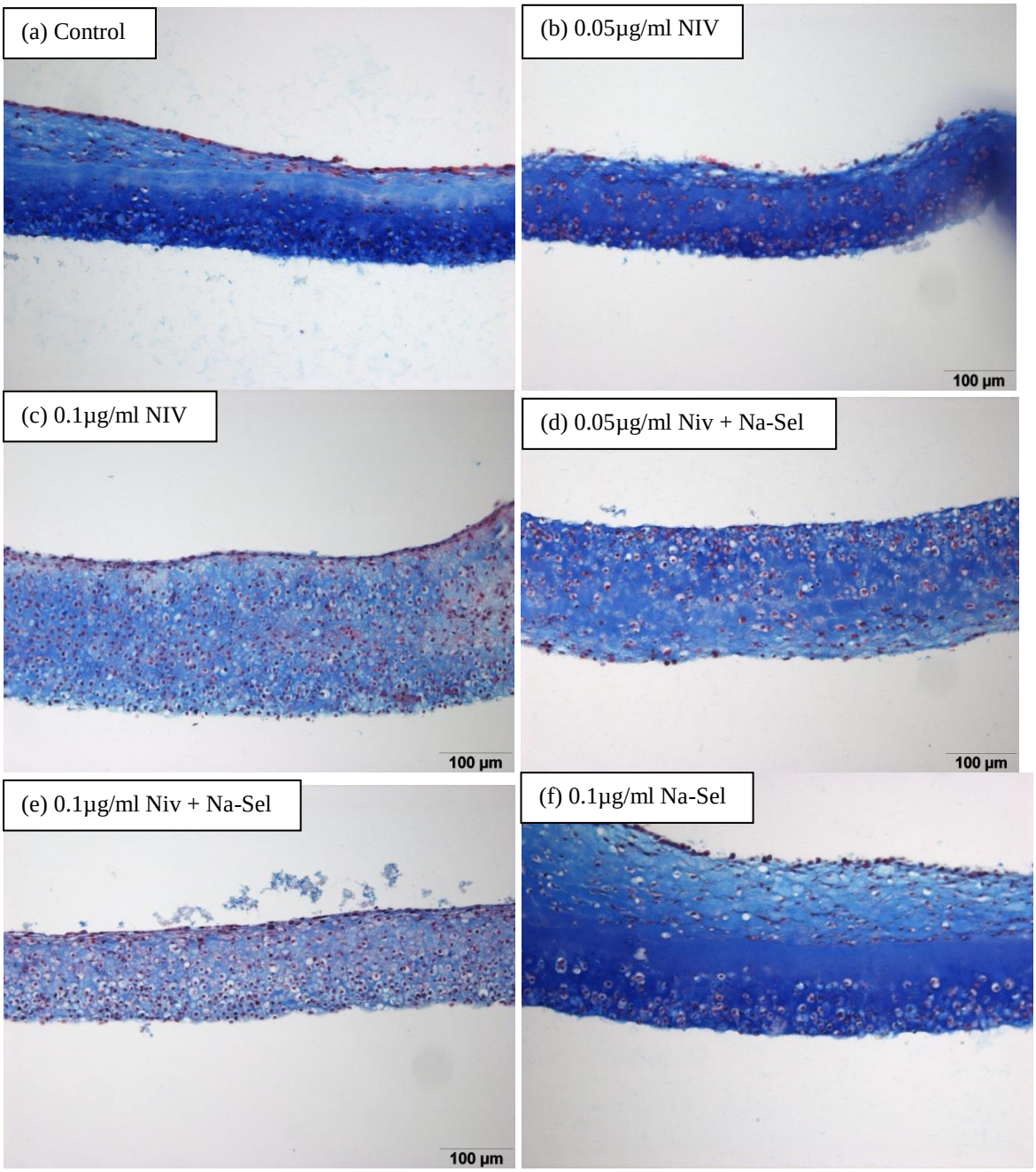


Figure 3.9 Histology staining with Alcian Blue and H&E for the different culture conditions: (a) Control, (b) 0.05µg/ml NIV, (c) 0.1µg/ml NIV, (d) 0.05µg/ml Niv + Na-Sel, (e) 0.1µg/ml Niv + Na-Sel and (f) 0.1µg/ml Na-Sel

In the presence of 0.05µg/ml sodium selenite (Na-Sel) and NIV (Fig 3.9d) the organisation of the cartilage zones more closely resembled that of the Control (compared to 0.1µg/ml NIV alone) containing 3 distinct layers, cellular shapes and staining intensities. In the presence of 0.1µg/ml Na-Sel and NIV (Fig 3.9e) the layers were fairly disorganised, however the zones are more recognisable than grafts cultured in the presence of 0.1µg/ml NIV alone. Grafts cultured in the presence of Na-Sel alone (Fig 3.9f) were very similar to the control.

3.9 Discussion

The objectives of the research presented in this chapter were to develop an *in vitro* culture system that mimics some of the characteristics of chondrocyte/cartilage pathology that is found in the pathogenesis of KBD. This was achieved with Transwell technology, using a modification of the method described by Kandel *et al*, 1995. The presence of the mycotoxin Nivalenol causes disruption of the chondrocytes within the Transwell neocartilage grafts, mimicking that seen in KBD leading to a reduction in graft weight, thickness and production of sGAG. The initial work on optimising doses also showed that higher doses of selenium had deleterious effects on these parameters, although not as severe as Nivalenol.

Once the doses of selenium (both Na-Sel and Sel-Met) and Nivalenol were optimised it was found that cellular metabolism (measured using the lactate assay) was fairly uniform for each treatment. However, cell viability (measured with the MTT assay) did show a decrease after week 2 in the Nivalenol groups. When combined with either selenium source there was less of a decrease, potentially demonstrating some protective benefit.

Grafts wet weights were adversely affected by the presence of Nivalenol, with significant decreases seen at both the high and lower dose. The presence of selenium had no significant effect, but with Na-Sel there was an increased trend, with the reverse being the case for Sel-Met. This was reflected in the result when Nivalenol and selenium were combined; with Na-Sel and Nivalenol the graft weight showed no significant difference to the control (although there was a decreased trend), whereas with Sel-Met and Nivalenol combined the results mirrored those of Nivalenol alone i.e. significant decrease compared to the control.

In summary, the work in this chapter has shown;

- Nivalenol has negative effects on cartilage graft development at all doses
- Selenium had negative effects only at high doses, but at lower doses there were no significant effects
- When Nivalenol was combined with sodium selenite there was an increase in graft weight when compared to Nivalenol alone, but this was not seen with selenium-l-methionine

CHAPTER 4

ANALYSIS OF THE BIOCHEMICAL PROPERTIES OF CARTILAGE GRAFTS AND METABOLITES FROM THE *IN VITRO* MODEL OF KASHIN BECK DISEASE

4.1 Introduction

The previous chapter demonstrated the use of an *in vitro* cartilage graft Transwell culture system. The development of the cartilage graft from bovine chondrocytes demonstrated the three histological distinct zones that resemble the superficial, middle and deep zones of native articular cartilage, and within these zones there were striking similarities in cell morphology and matrix organisation to that of native articular cartilage. By using this established Transwell culture system, the previous chapter developed an *in vitro* culture system that mimics cartilage pathology found in KBD *in vivo.*

Kashin-Beck disease (KBD) is an endemic osteoarthropathy with pathological changes occurring in growth plate and articular cartilage in humans. It manifests as cartilage degeneration and necrosis. It is postulated that KBD is due to fungal mycotoxins (such as nivalenol) infiltrating the diet and a regional selenium deficiency in the environment providing food sources in a broad belt across China.

Previous work, described in Chapter 3, established an *in vitro* culture system in which chondrocytes are cultured to produce an *in vitro* cartilage graft. Subjecting these chondrocytes to either selenium (SEL), nivalenol (NIV) or in combination during the growth of the graft was found to alter the biochemical composition and morphology of the cartilage graft. In addition, NIV was shown to affect cartilage metabolism and the organisation of proteoglycans and subsequently the extracellular matrix. It was also demonstrated that SEL had a protective effect by reducing the effects of NIV on the cartilage graft development, metabolism and morphology in a dose dependent manner.

Thus, the primary aim of studies described in this chapter was:-

Aim To develop an *in vitro* model to study the effect of different sources of selenium (i.e. sodium selenite and selenium-l-methionine) and nivalenol (a mycotoxin) on chondrocyte/ cartilage metabolism. These studies involved analyses of the two different selenium sources and nivalenol alone and in combination.

Figure 4.1 below shows a schematic overview of general methods and an outline of analysis undertaken resulting in the data presented in this chapter.

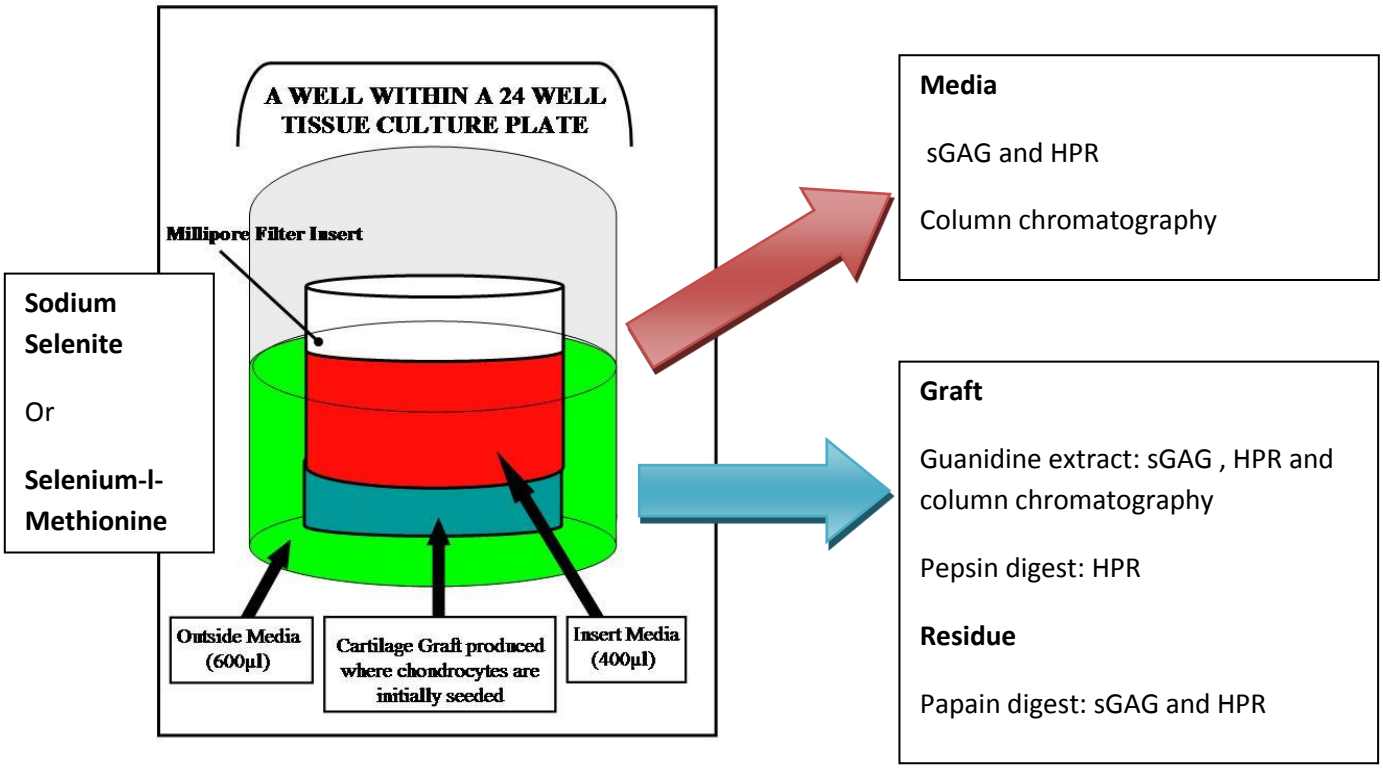


Figure 4.1 Diagram showing a basic outline of the methodology used to provide the results given in this chapter

4.2 The effect of selenium (SEL), Nivalenol (NIV) or in combination on media sGAG content.

Chondrocytes were isolated from 7 day old bovine articular cartilage following pronase and collagenase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly, with media from the insert being stored and pooled on a weekly basis. The media was assessed for sGAG content using the DMMB assay (section 2.13) and are shown in Figure 4.2.

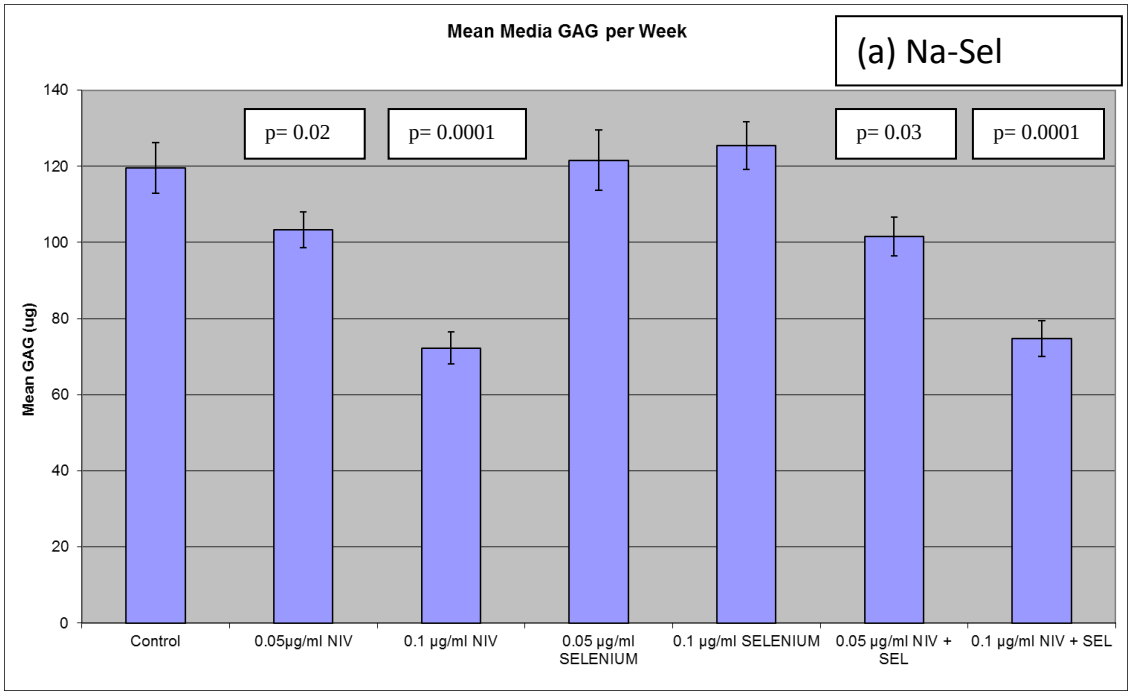
Figure 4.2 demonstrates that the sGAG content of the media significantly decreased with the addition of Nivalenol in a dose dependant manner. Both selenium compounds at each dose had showed no statistical difference to the Control. Neither selenium compound showed a protective effect when combined with Nivalenol.

Figure 4.3 shows that the media sGAG produced each week was fairly consistent irrespective of the treatment in both experiments.

4.3 The effect of selenium (SEL), Nivalenol (NIV) or in combination on Graft sGAG content of the Extract (4M Guanidine HCl)

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly. At the end of the 4 week culture period the graft was harvested and proteoglycan extracted with 4M Guanidine HCl (section 2.10) and analysed for sGAG content. Figure 4.4 shows that the effect of Nivalenol on sGAG content in the cartilage extracts are similar to that seen in the media, with a statistically significant decrease when compared to the control in a dose dependant manner.

Sodium selenite had no significant effect on sGAG content at the lowest dose, but did show a decrease at the higher dose. Its presence did not infer any protective effect when used in combination with Nivalenol. Selenium-l-methionine appeared to lower the sGAG content at both doses but this was not statistically significant. Its presence did not have protective effect when used in combination with Nivalenol.



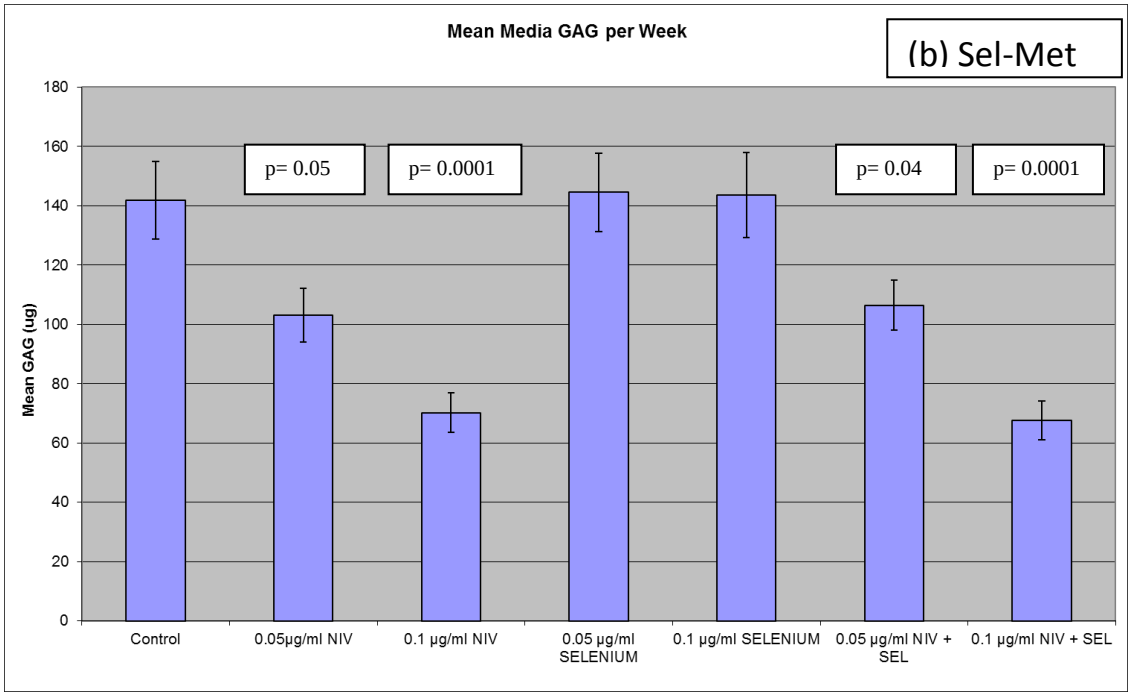


Figure 4.2 Mean weekly media sGAG with (a) Sodium Selenite and (b) Selenium-l-methionine (n=9)

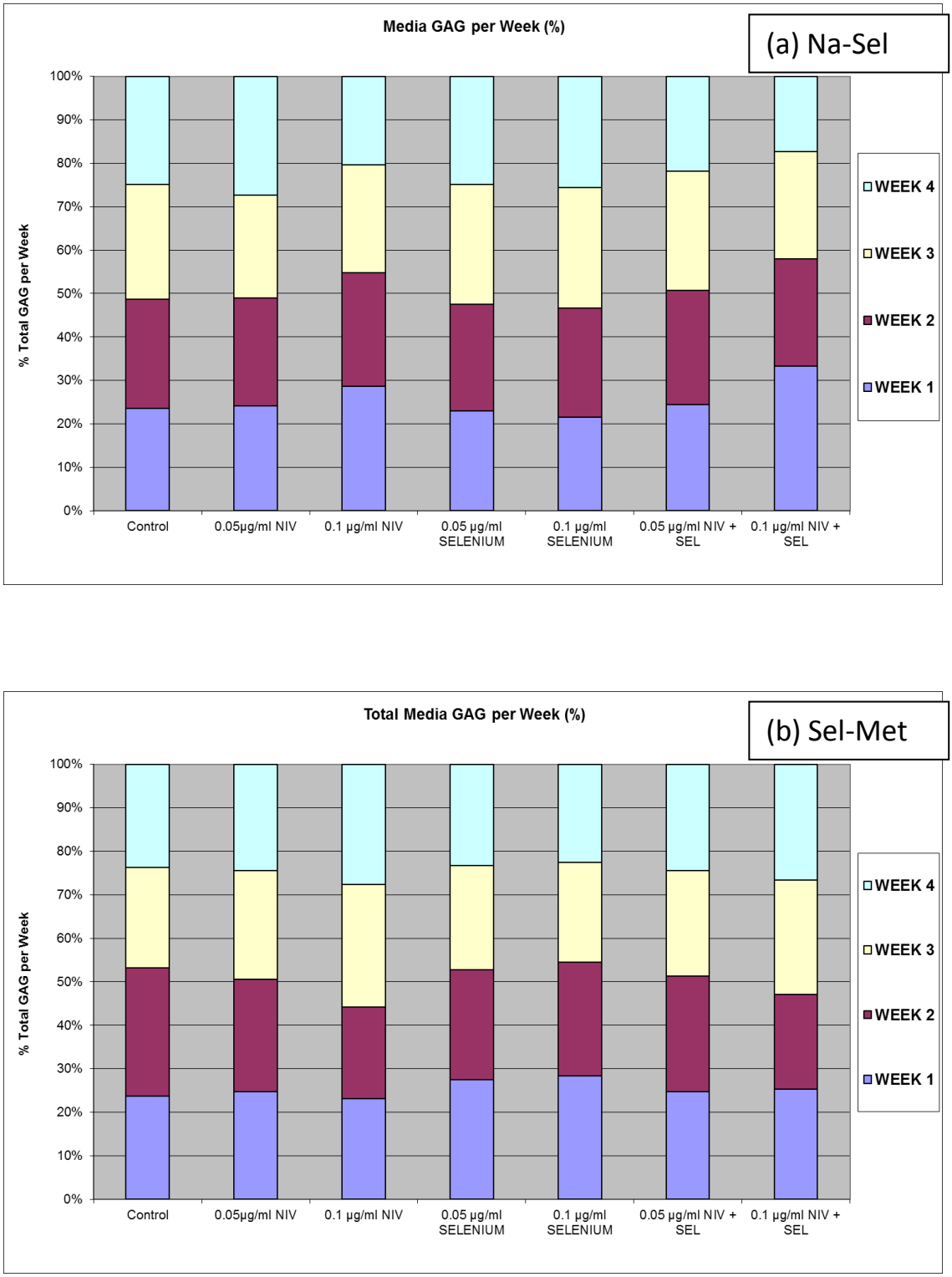
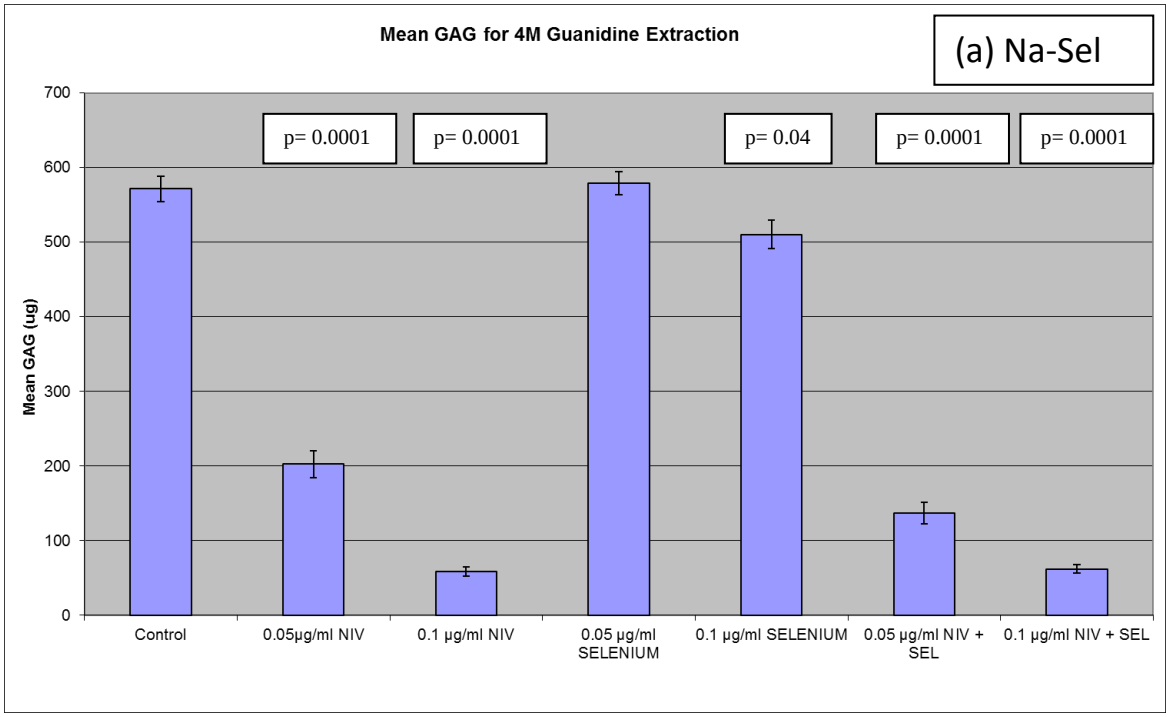


Figure 4.3 Total sGAG present in the culture media expressed as a weekly percentage with (a) Sodium Selenite and (b) Selenium-l-methionine



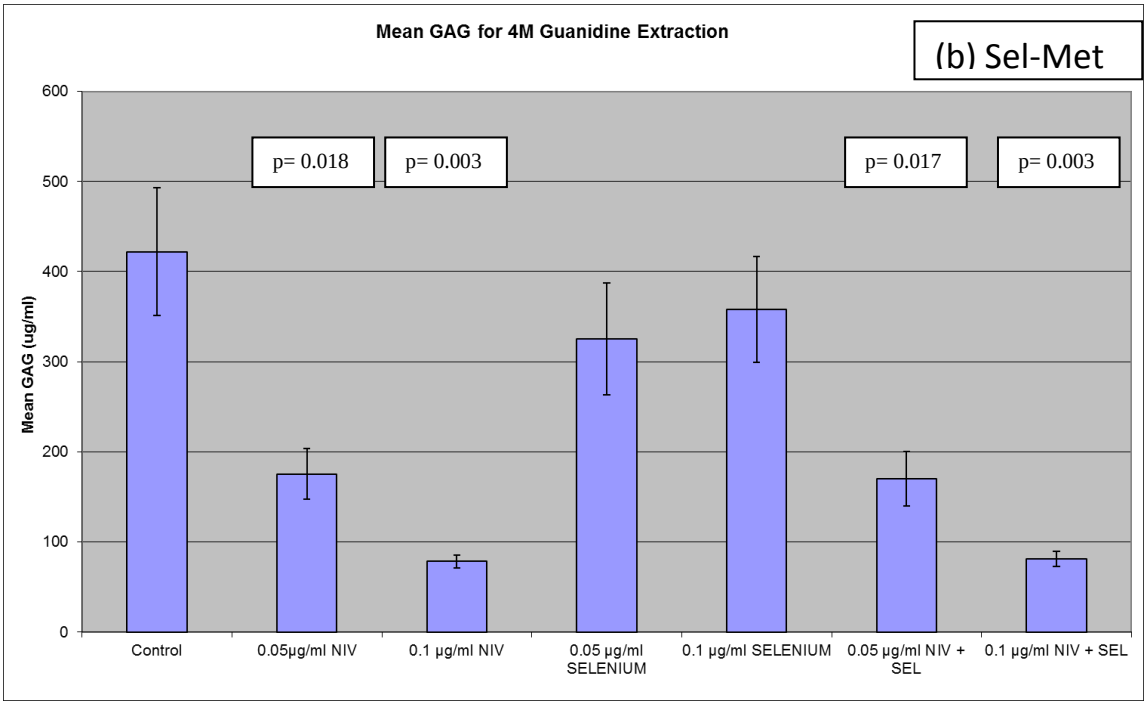


Figure 4.4 Graft sGAG content following 4M Guanidine extraction of the graft with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8)

4.4 The effect of selenium (SEL), Nivalenol (NIV) or in combination on sGAG content of the Graft Residue (Papain digest)

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly. At the end of the 4 week culture period the graft was harvested and proteoglycan extracted with 4M Guanidine HCl leaving a residue that was then digested with Papain (section 2.12) and analysed for sGAG content.

Figure 4.5 shows that Nivalenol caused a decrease in sGAG content compared to the control in a dose dependant manner in both experiments but this did not reach statistical significance in the selenium-l-methionine experiment which probably relates to the spread of the data (note the large standard errors).

There were significant decreases with Nivalenol present in the sodium selenite experiment. Neither selenium compound had a protective effect when used in combination with NIV.

4.5 The effect of selenium (SEL), Nivalenol (NIV) or in combination on Total sGAG content.

Figures 4.6 and 4.7 show similar results are seen with both selenium experiments, with a general trend showing that an increased Nivalenol concentration decreases sGAG production. All groups containing Nivalenol had significantly less total sGAG production than the control, whereas the addition of either selenium compound (at either dose) had no significant effect.

In the control and selenium alone groups the proportion of sGAG released into the media was approximately 50%, but when Nivalenol was added this proportion increased to 60-70%. Again, it appears that the addition of either selenium to Nivalenol infers no protective benefit.

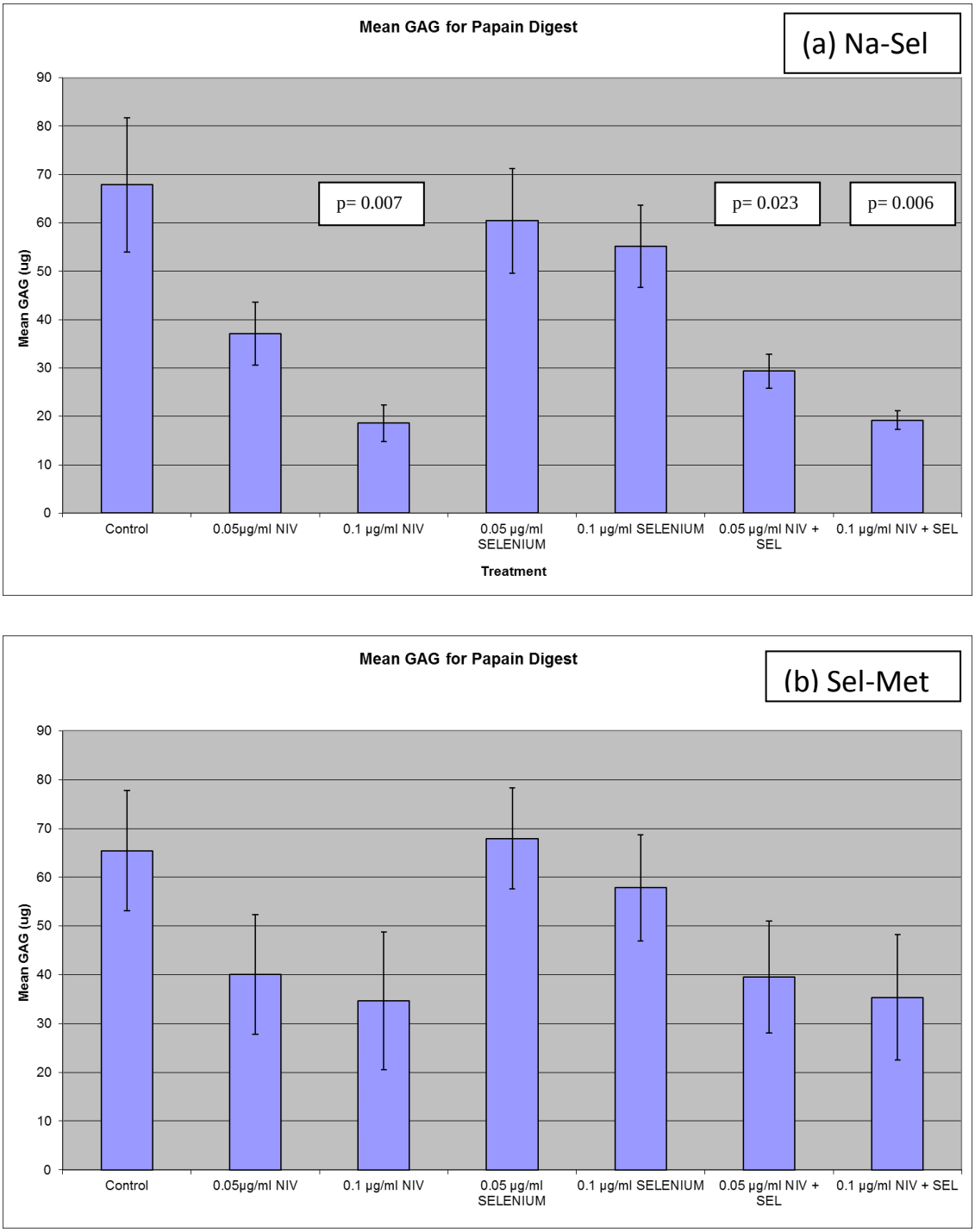
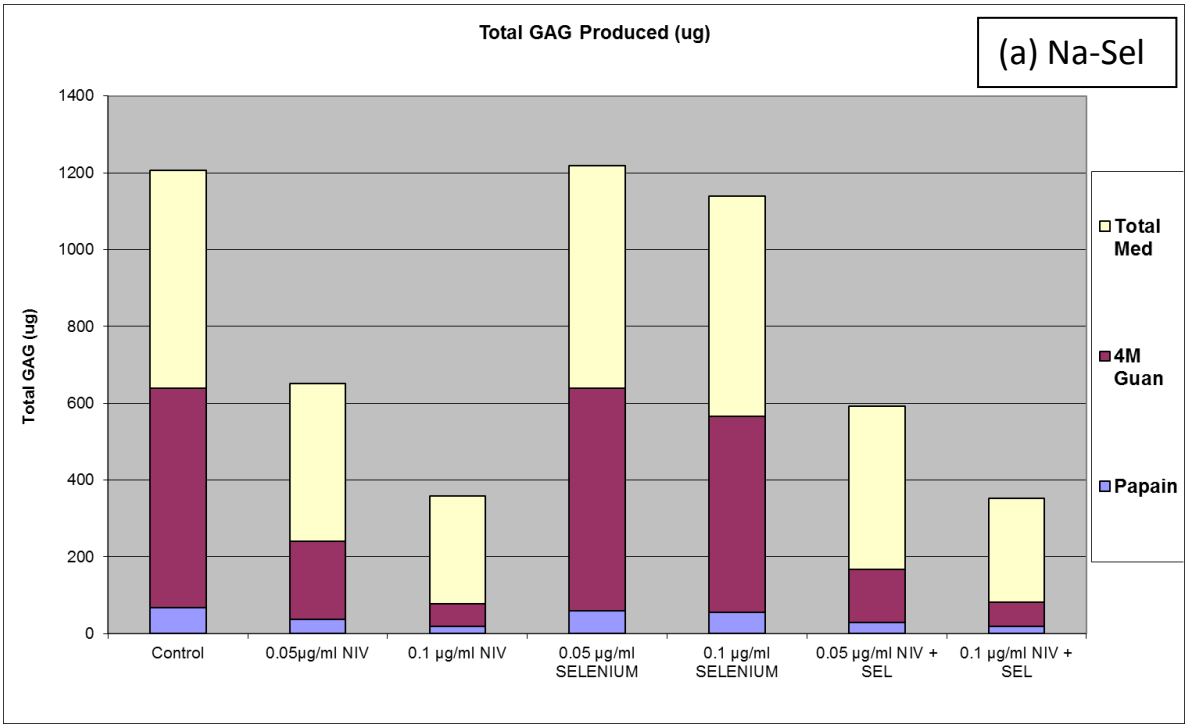


Figure 4.5 sGAG content following Papain digest of the residue with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8)



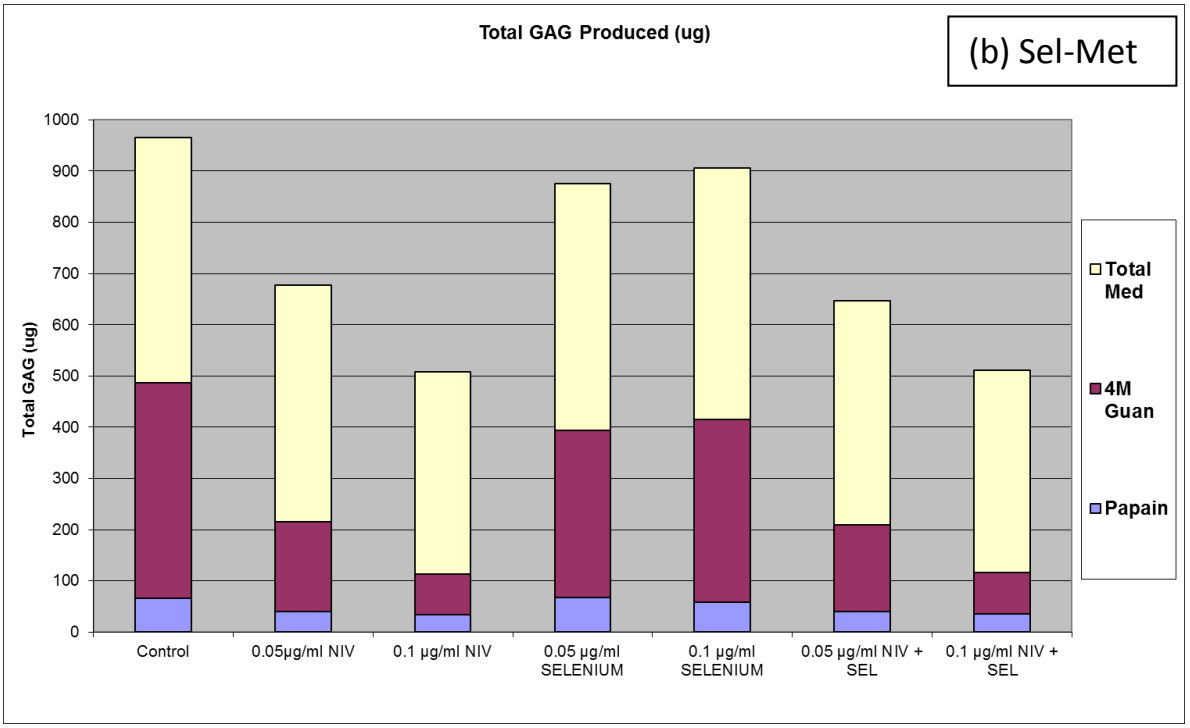
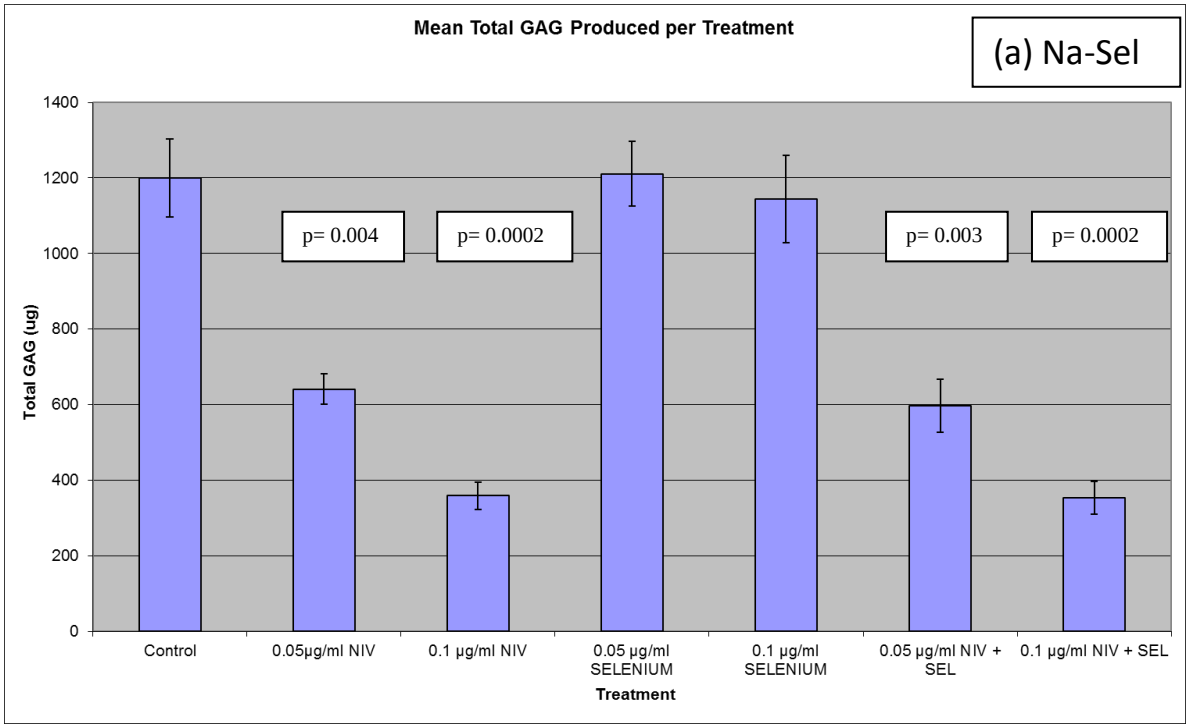


Figure 4.6 Total sGAG produced demonstrating breakdown of proportions in the graft and the media with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8/9)



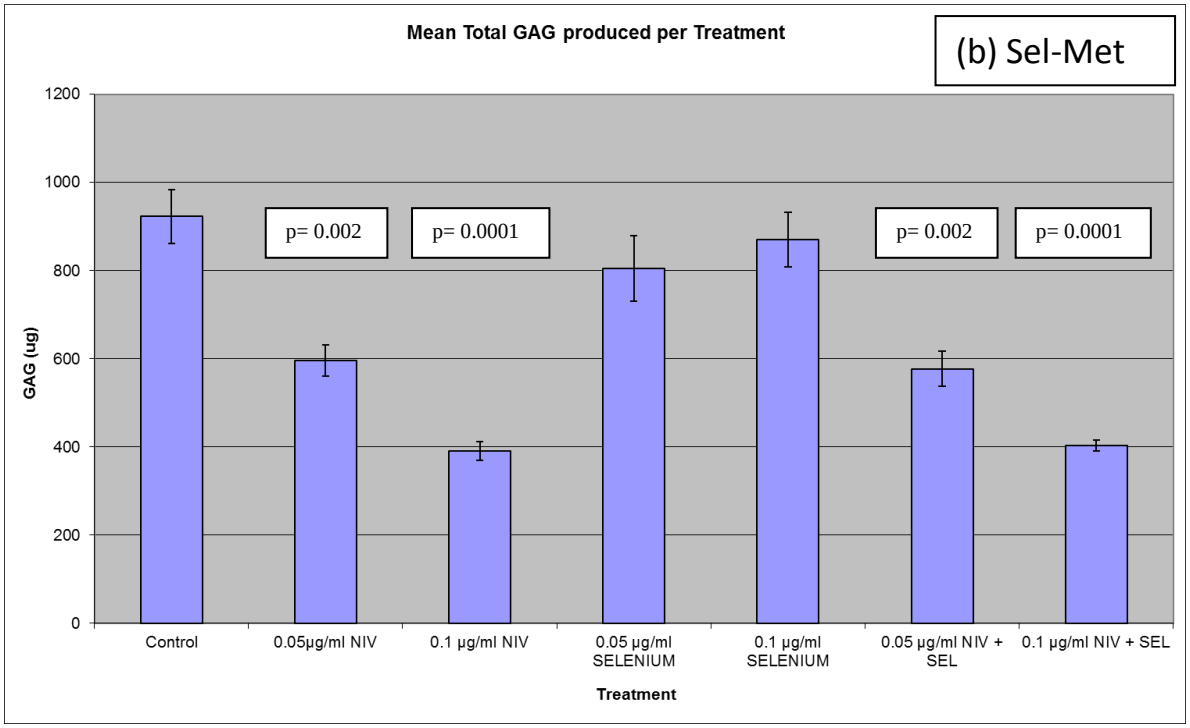


Figure 4.7 Mean Total sGAG produced with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8/9)

4.6 The effect of selenium (SEL), Nivalenol (NIV) or in combination on media collagen content.

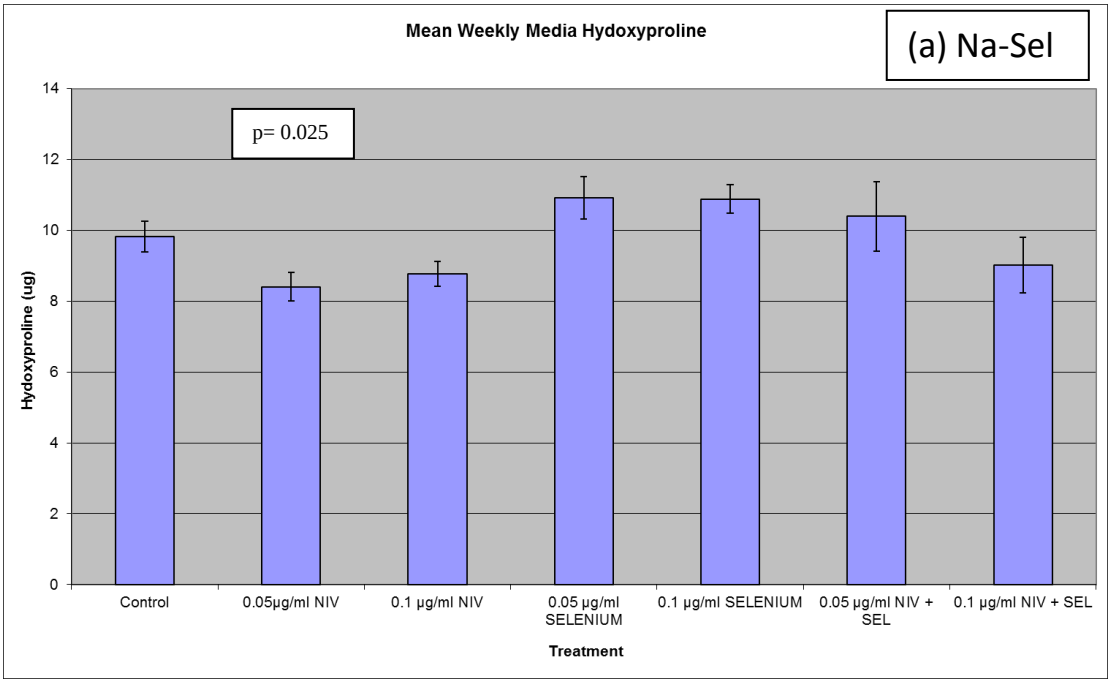
Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4 x 10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly, with media from the insert being stored and pooled on a weekly basis. The media was assessed for collagen content using the hydroxyproline assay (section 2.15)

Figures 4.8 and 4.9 show that as with sGAG, the proportional effects at each of weeks 1, 2, 3 and 4 appear to be about the same for each different treatment, and for each type of selenium. The presence of Nivalenol reduced the hydroxyproline released into the media, but it seemed to have less of an effect on collagen than it did for sGAG. In the sodium selenite experiment the only treatment that reached a statistical significant difference to the control was 0.05µg/ml Nivalenol, although both treatments containing the higher dose of Nivalenol do show a notable decrease in media hydroxyproline. In the selenium-l-methionine experiment, all treatments containing Nivalenol were significantly lower than the control, with selenium not showing a protective effect.

4.7 The effect of selenium (SEL), Nivalenol (NIV) or in combination on collagen content of the Graft Extract (Pepsin)

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4 x 10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly. At the end of the 4 week culture period the graft was harvested and collagen extracted with pepsin (section 2.11) and analysed using the hydroxyproline assay. Results are shown in Figure 4.10. In both experiments, Nivalenol groups showed a significantly decreased graft collagen content than the control (seen in a dose dependant manner in the Se-Met experiment).

In both experiments, the higher selenium dose showed a significant increase in collagen compared to the control, with the lower dose being more or less equal. The addition of either selenium to Nivalenol did not show any convincing protective effect.



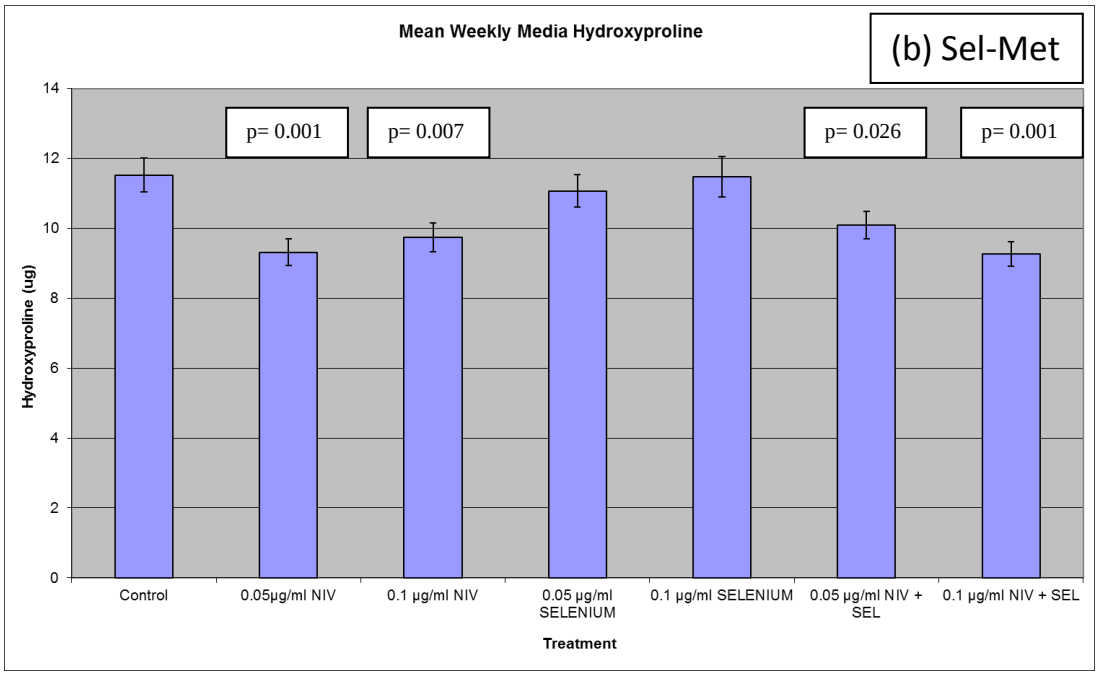
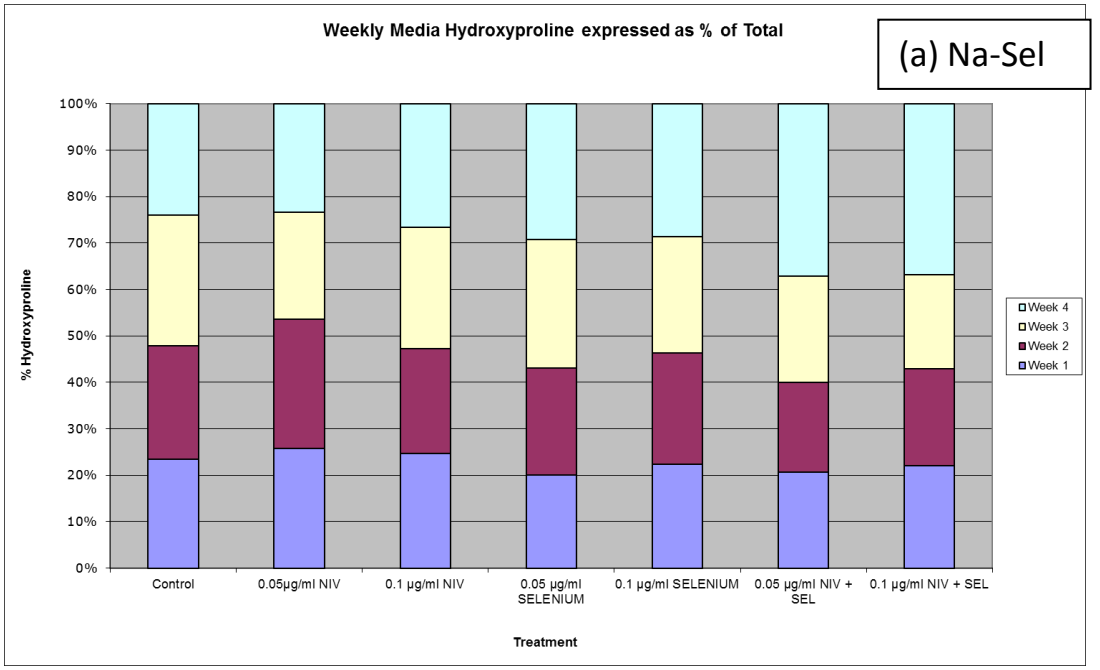


Figure 4.8 Mean weekly hydroxyproline present in the culture media with (a) Sodium Selenite and (b) Selenium-l-methionine (n=9)



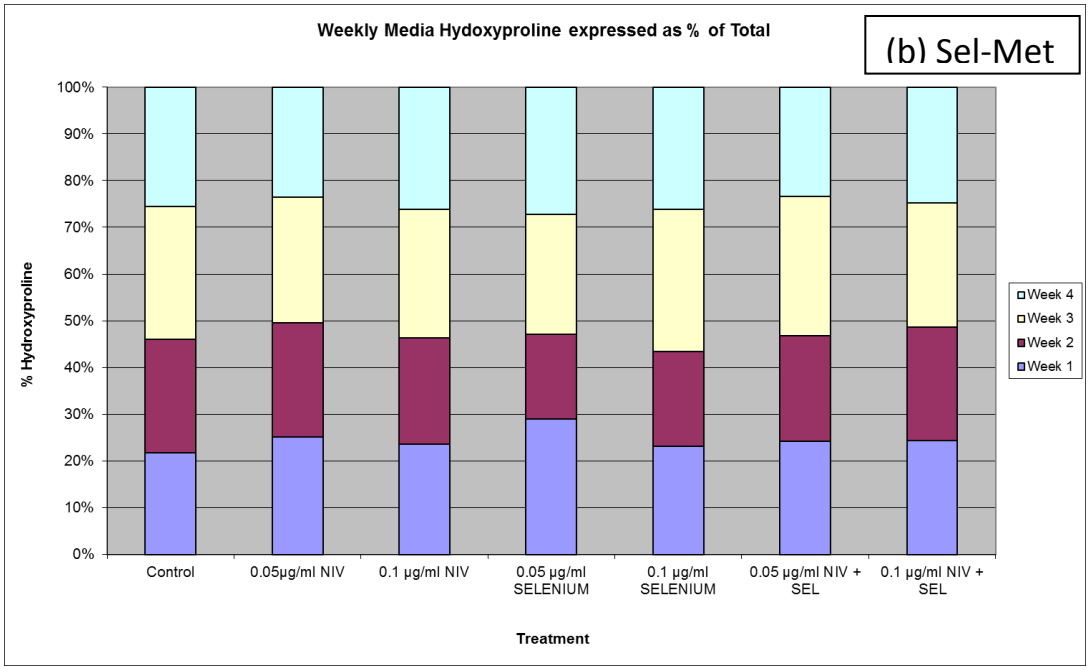
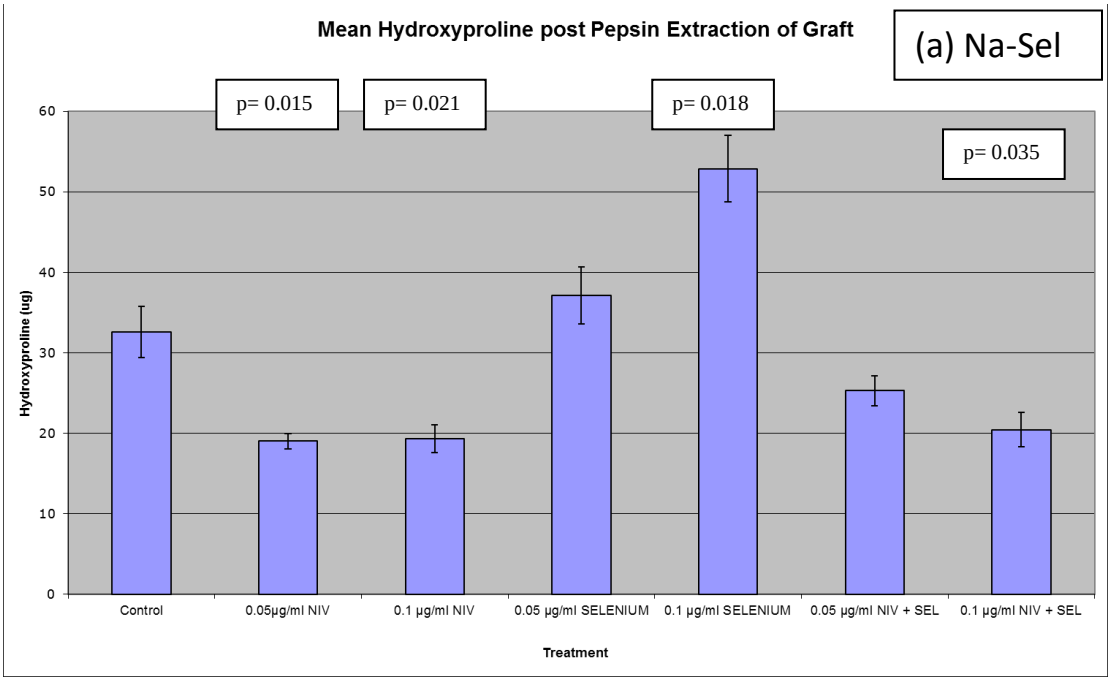


Figure 4.9 Total Hydroxyproline present in the culture media expressed as a weekly percentage with (a) Sodium Selenite and (b) Selenium-l-methionine (n=9)



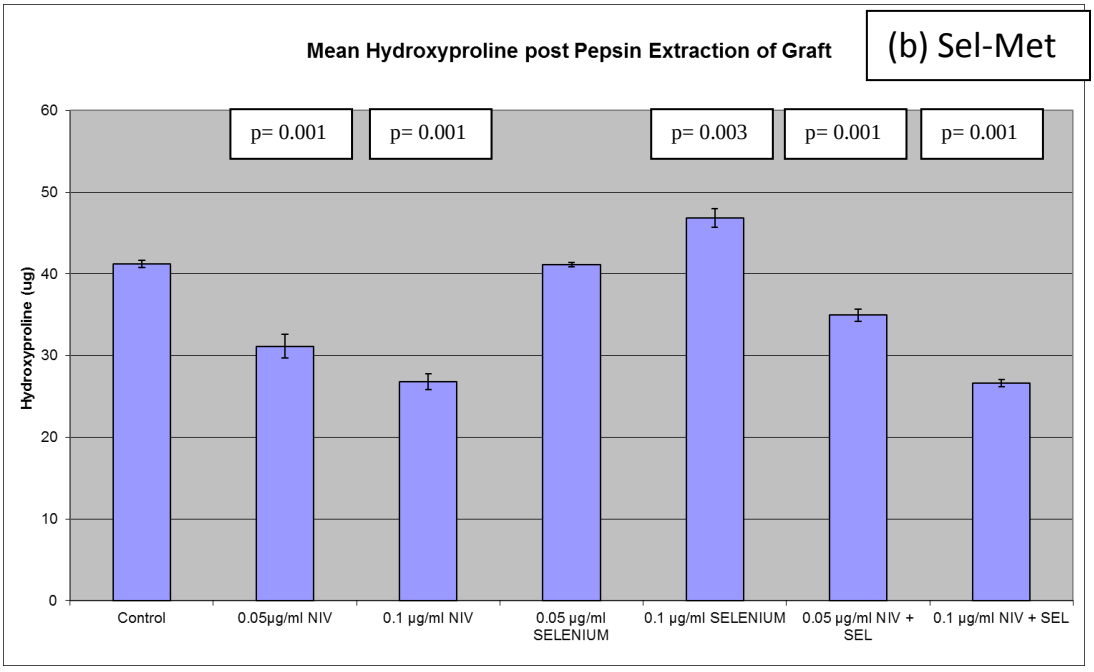


Figure 4.10 Mean total hydroxyproline following Pepsin extraction of the graft with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8)

4.8 The effect of selenium (SEL), Nivalenol (NIV) or in combination on collagen content of the Graft Residue (Papain digest)

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly. At the end of the 4 week culture period the graft was harvested and collagen extracted with Pepsin leaving a residue that was then digested with Papain (section 2.12) and analysed for using the hydroxyproline assay. Results are shown in Figure 4.11

In both experiments, Nivalenol groups showed a significant decrease in collagen content compared to the control in a dose dependant manner, with no protective effect seen on addition of either selenium.

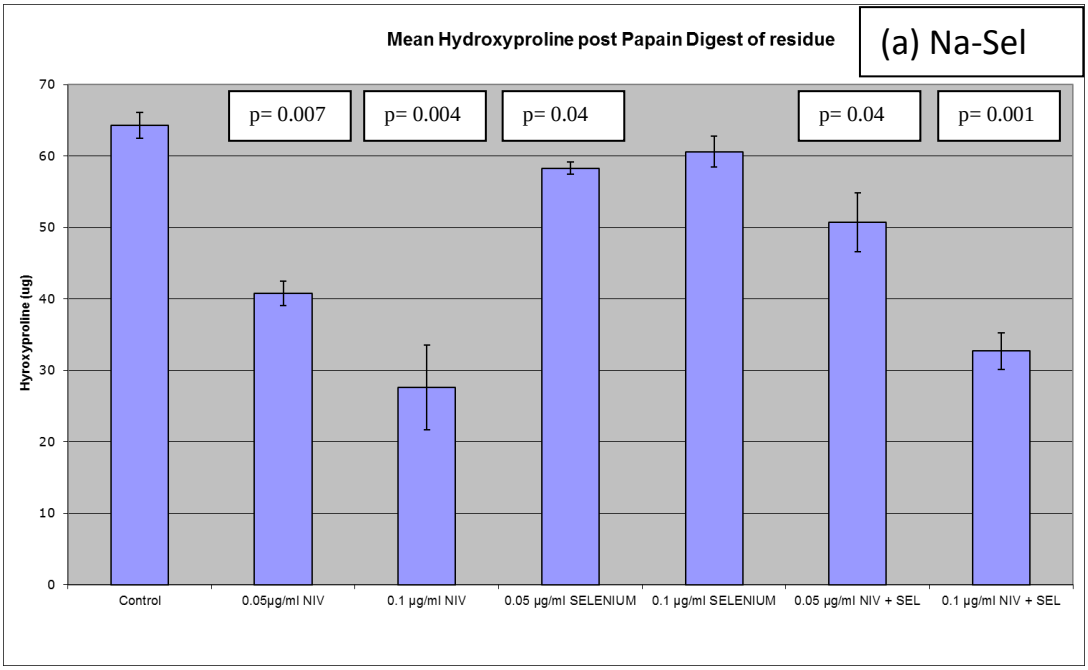
In the selenium groups the collagen content was comparable with that of the control, however the lower dose of sodium selenite was found to be statistically significantly lower than the control, although this decrease was markedly less than seen with Nivalenol groups.

4.9 The effect of selenium (SEL), Nivalenol (NIV) or in combination on Total Collagen content.

The results for total collagen synthesized are shown in Figures 4.12 and 4.13 and are almost identical for both experiments. Nivalenol was found to decrease total collagen in a dose dependant manner, and these results were statistically significant. As with sGAG, the addition of either selenium to Nivalenol did not show a protective effect.

In selenium groups the results were comparable to the control, and in the higher selenium dose there appears to be an increase in total collagen with sodium selenite at 0.1µg/ml show a statistically significant increase.

When the total collagen is broken down into its component parts, the majority is retained in the graft for all treatments. However in the control and selenium groups this is higher (between 60-70%) than in the Nivalenol and combined treatments (between 50-60%).



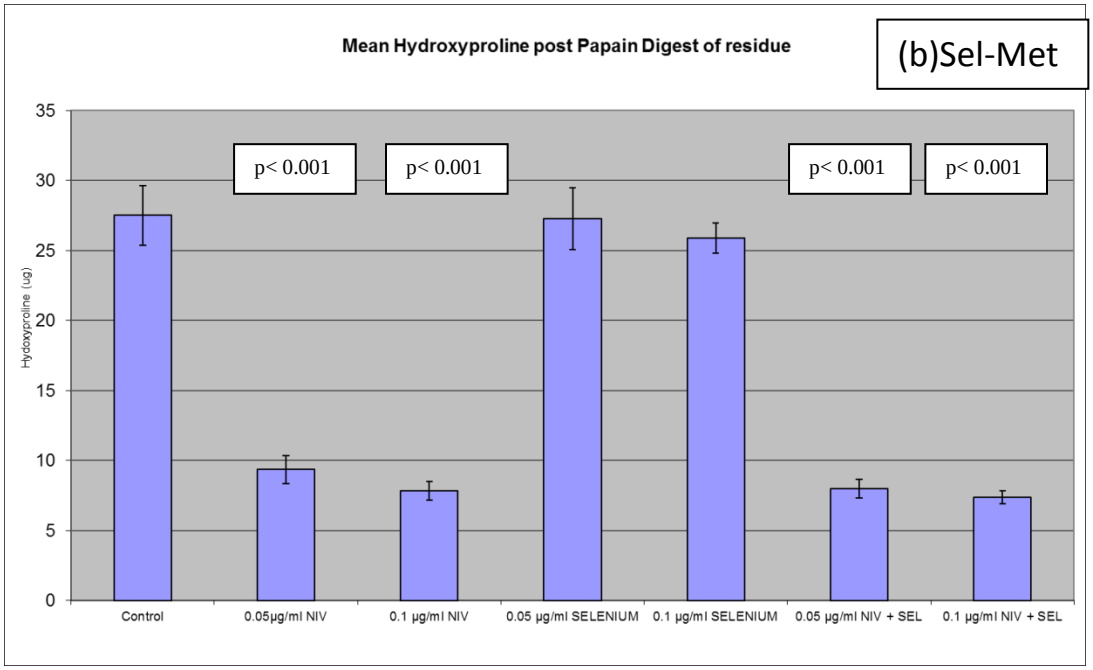
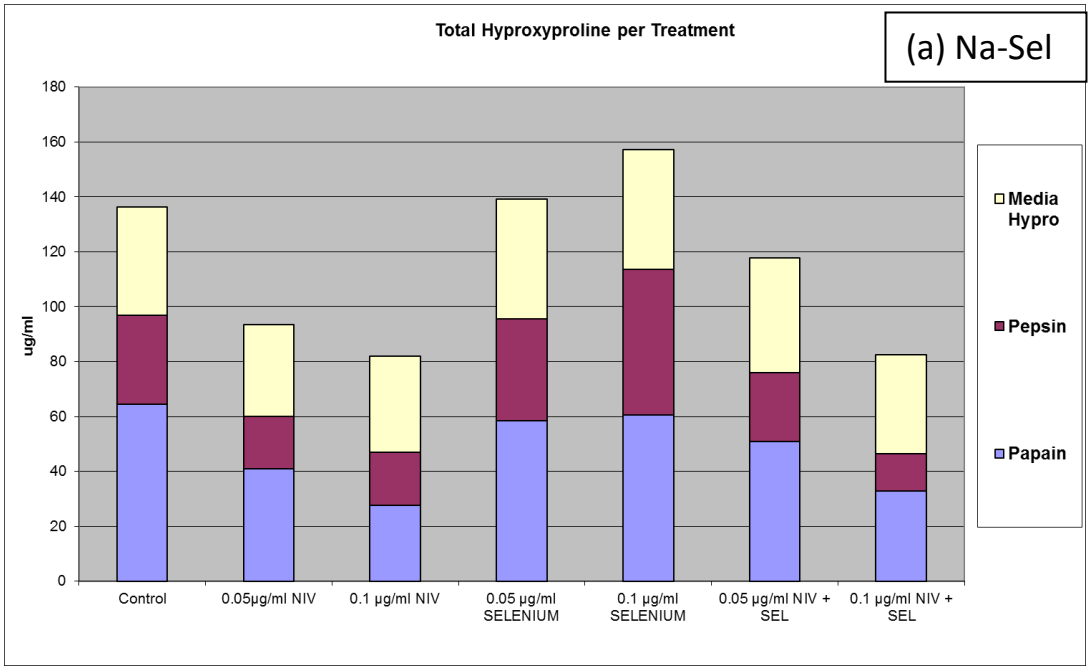


Figure 4.11 Mean total hydroxyproline following Papain digest of the residue with (a) Sodium Selenite and (b) Selenium-l-methionine. (n=8)



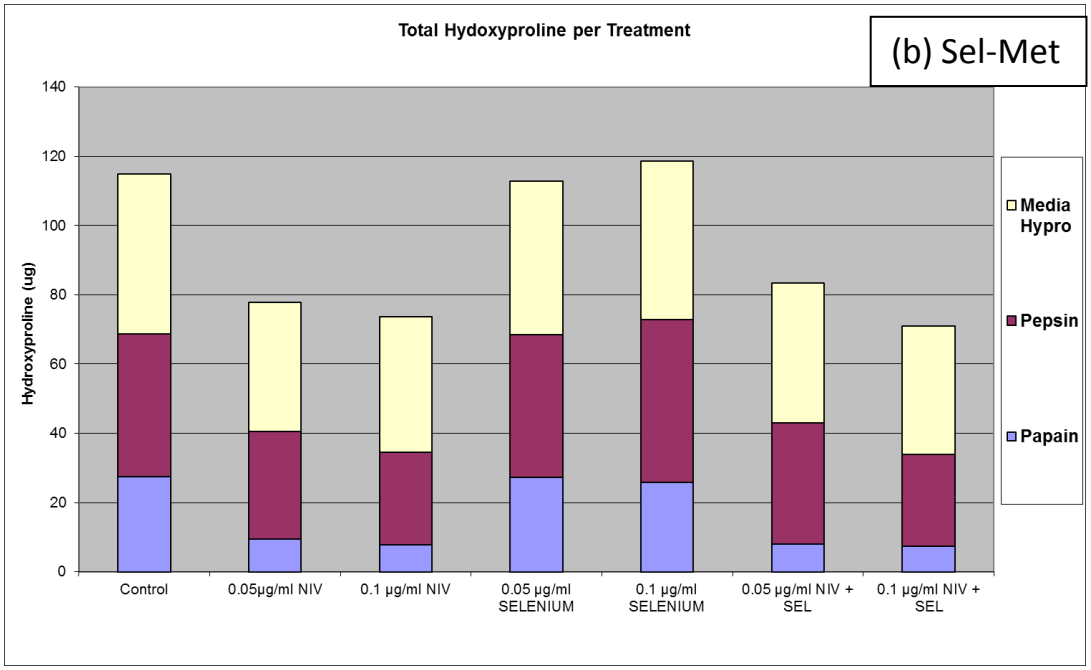
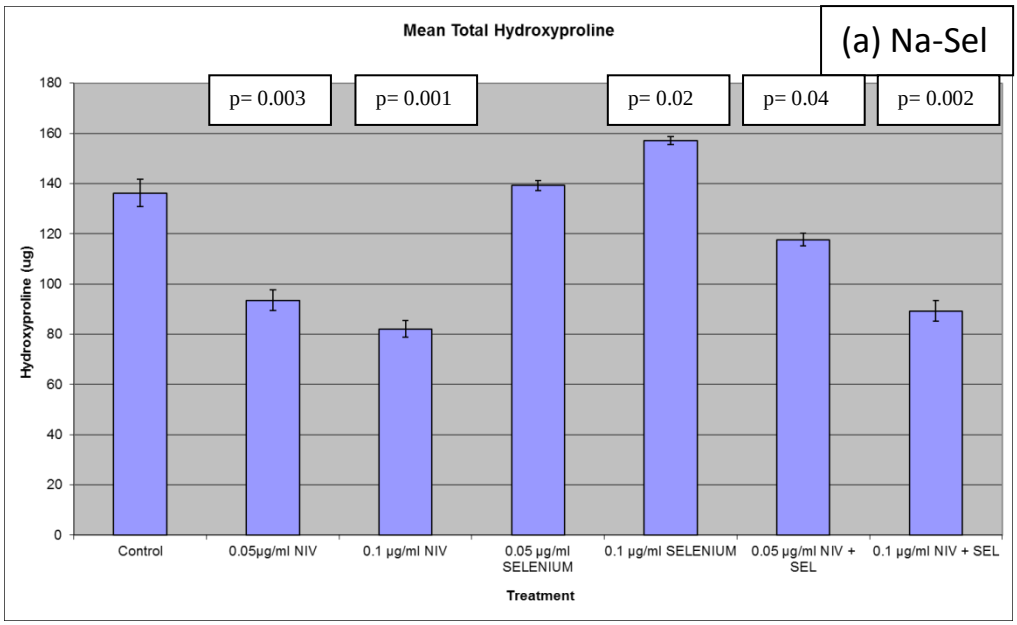


Figure 4.12 Total Hydroxyproline produced demonstrating breakdown of proportions in the graft and the media with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8/9)



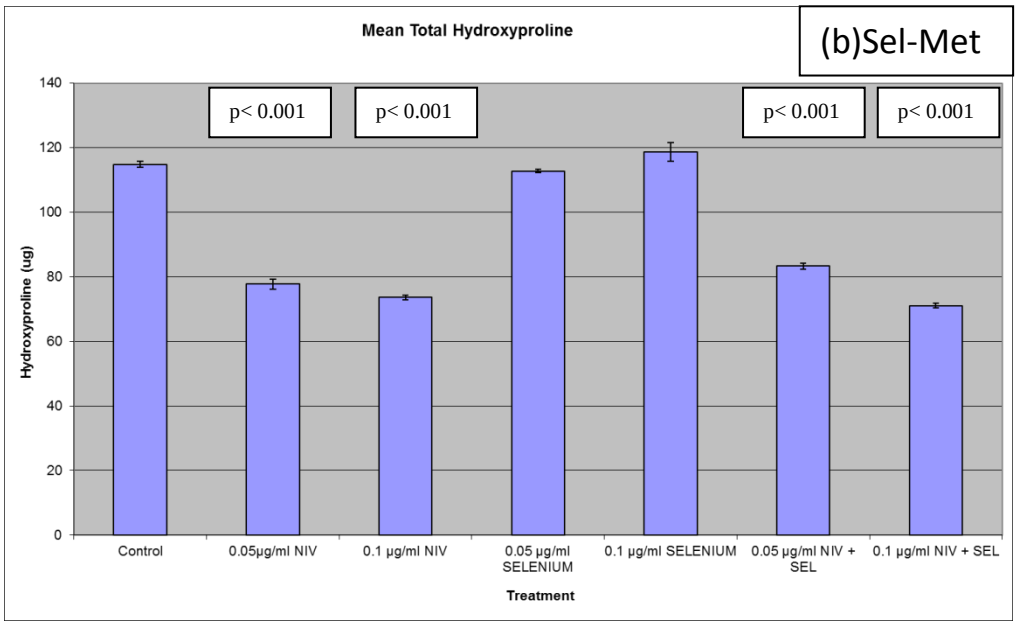


Figure 4.13 Total Hydroxyproline produced with (a) Sodium Selenite and (b) Selenium-l-methionine (n=8/9)

4.10 Analysis of Cartilage Aggregating Proteoglycan- Aggrecan; The effect of Selenium (SEL), Nivalenol (NIV) or in combination on aggrecan aggregation in the media

All results for this section are for sodium selenite only. The column used was a Sepharose CL-2B to distinguish between aggrecan in HA/link protein bound aggregates from non- aggregated aggrecan.

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly, with media from the insert being stored and pooled on a weekly basis. The media was assessed for sGAG content using the DMMB assay. 2mls of media for each treatment was run through the CL-2B column to assess the molecular composition (section 2.16). Before processing samples from the experiment, standards for aggregated aggrecan and monomer were processed for reference comparison.

Figure 4.14(a) shows a majority of sGAG is in large aggrecan aggregates that are excluded from the CL-2B column. Figure 4.14(b) shows a majority of sGAG for aggrecan monomers are found in the included volume of the CL-2B column. The results, shown in Figure 4.15, for each treatment are very similar, showing the presence of a majority of large aggrecan aggregates. This suggests therefore that selenium and Nivalenol had little or no effect on aggrecan aggregation of the proteoglycan released into the media.

4.11 Analysis of Cartilage Aggregating Proteoglycan- Aggrecan; The effect of Selenium (SEL), Nivalenol (NIV) or in combination on aggrecan aggregation in the graft

Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion. 4×10^6 cells were seeded into Millipore Transwell inserts within 24 well plates. Media was changed thrice weekly. At the end of the 4 week culture period the graft was harvested and proteoglycan extracted with 4M Guanidine HCl (section 2.5.1) and analysed for sGAG content. One or 2ml (depending on sGAG content) of solution for each treatment was run through the CL-2B column to assess the molecular composition (section 2.6.4). Figures 4.16 (c)-(e) show the majority of aggrecan in the graft in these

treatments was in the form of large aggregates, but there was more monomer present than seen in the media.

The ‘flat’ profile seen for Nivalenol 0.1µg/ml may be due to only a small quantity of sGAG being loaded (as there was only small quantities yielded following 4M Guanidine extraction).

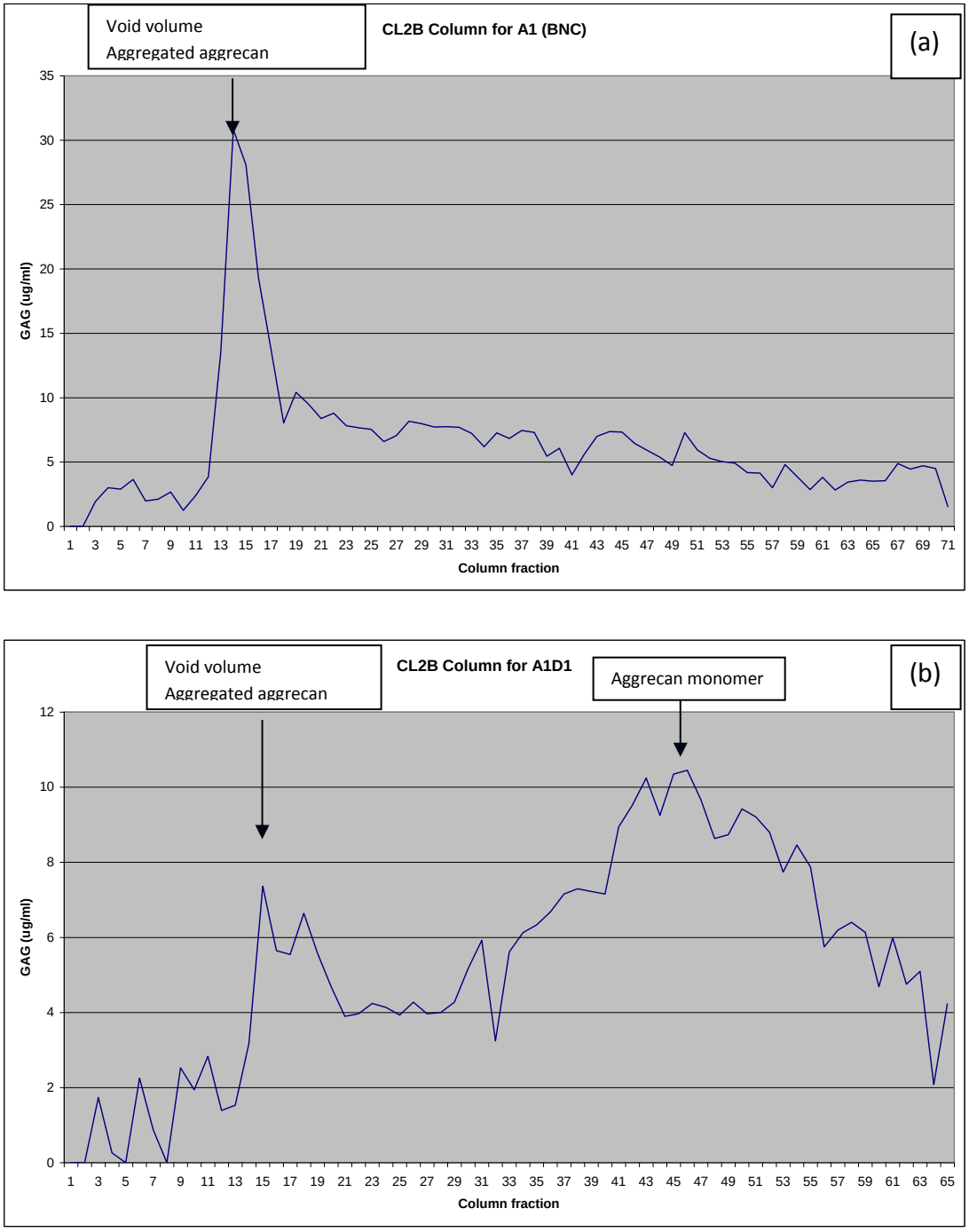


Figure 4.14 Column chromatography profiles for (a) Bovine A1 Standard (Aggregate) and (b) Bovine A1D1 Standard (Monomer)

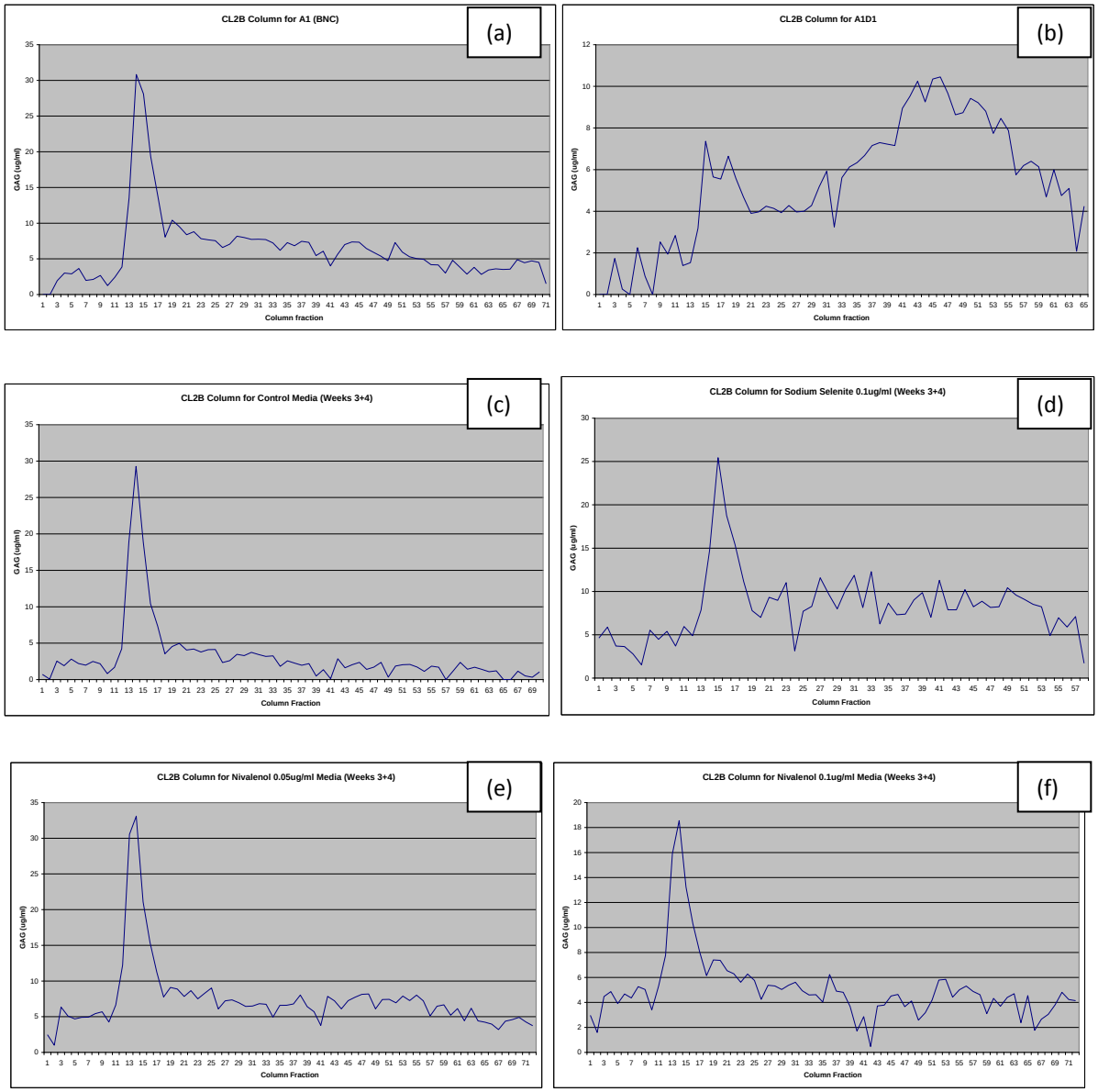


Figure 4.15 Column chromatography profiles for (a) Bovine A1 Standard (Aggregate), (b) Bovine A1D1 Standard (Monomer) and media samples for (c) Control, (d) Sodium selenite 0.1µg/ml, (e) Nivalenol 0.05µg/ml and (f) Nivalenol 0.1µg/ml

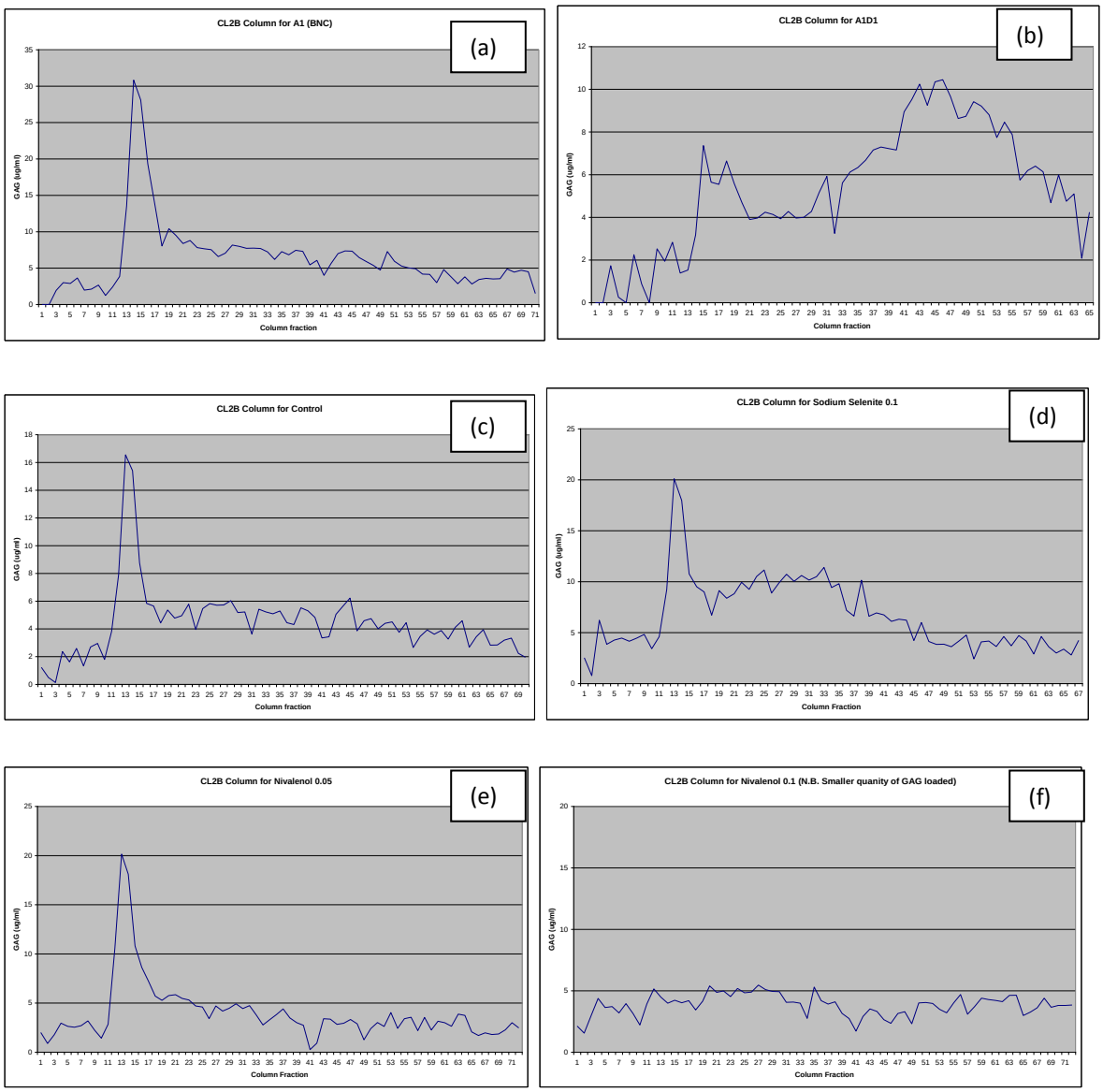


Figure 4.16 Column chromatography profiles for (a) Bovine A1 Standard (Aggregate), (b) Bovine A1D1 Standard (Monomer) and 4M Guanidine extract samples for (c) Control, (d) Sodium selenite 0.1µg/ml, (e) Nivalenol 0.05µg/ml and (f) Nivalenol 0.1µg/ml

4.12 Discussion

The objectives of the research presented in this chapter were to produce an *in vitro* model of KBD to study the intricate changes that were occurring in cellular and matrix metabolism. This was achieved with Transwell technology, using a modification of the method described by Kandel *et al*, 1995.

The amount of sGAG synthesized by the cartilage graft was assessed by analysing the media and the graft itself. The sGAG released into the media in the presence of NIV (either alone or combined with either selenium source) was decreased compared to control groups in a dose dependant manner. In the presence of either selenium compound there was no change in sGAG found in the media (Figure 4.2).The sGAG in graft Guanidine HCl extract was significantly decreased in a dose dependent manner with NIV. With either selenium source a decreased trend was observed for both concentrations, but this was only significant for Na-Sel at the highest dose. With a combination of NIV and either selenium results followed the trend of NIV alone (Figure 4.4). The sGAG in the graft residue showed a decreased trend with NIV (but this was only significant at the highest dose). There was no significant change seen with either selenium source. When NIV was combined with either selenium the results followed the trend of NIV alone (Figure 4.5). Collectively, figures 4.6 and 4.7 shows a significant sGAG decrease with NIV in a dose dependant manner, with the results being the same when NIV and either selenium source were combined. There was no change seen with either selenium source at either dose.

We also analysed hydroxyproline deposition in these experimental systems. Hydroxyproline can be used as a surrogate for total collagen; approximately 13.5% of collagen is composed of hydroxyproline, therefore in order to convert hydroxyproline to collagen one can simply multiply the value by 7.41. In the media there were small but significant differences in the collagen content of grafts exposed to NIV. However, there was no significant difference with either selenium source. When NIV and selenium sources were combined the decreases were similar to NIV alone (Figure 4.8). In the cartilage grafts the collagen content was decreased with NIV alone and NIV with either selenium source. In contrast, grafts cultured with either selenium showed a significantly increased collagen content at the highest selenium doses (Figure 4.10). With the graft residues, NIV or NIV with selenium showed a decreased collagen content. For either selenium the collagen contents were unchanged compared to the control (Figure 4.11). The composite analysis (i.e.

media+extract+residue) of collagen production of the cartilage grafts were significantly decreased with NIV or NIV and selenium exposure. In contrast either selenium source demonstrated a trend towards increased collagen content with only Na-Sel at the highest dose showing a significant increase (Figures 4.12 and 4.13). Collectively, these studies indicate that NIV exposure significantly affects both sGAG and collagen biosynthesis and that when NIV is in combination with either selenium source there is no protective effect. Nevertheless, our studies show that addition of either source of selenium has no significant effects on sGAG biosynthesis, but both selenium sources showed a trend towards increased collagen biosynthesis with just the highest dose of Na-Sel showing a significant difference.

We also investigated the macromolecular characteristics of aggrecan aggregates in some of these different experimental culture systems. Analysis of some of aggrecan aggregates released into the culture media (from control, Na-Sel and NIV) showed no major changes in aggrecan aggregation (Figure 4.15). Note: because of reduced yields only some of the samples could be analysed. However, analysis of aggrecan aggregates in the cartilage grafts showed normal aggregation in the control and NIV at low doses, but increased non-aggregating proteoglycan at high dose selenium and almost no aggregation in high dose NIV, although this may have been due to the small quantity loaded (Figure 4.16). Collectively, our analyses of an incomplete set of samples showed no loss of aggregation of aggrecan aggregates present in the media but some increase in non-aggregating aggrecan in high doses of Na-Sel and NIV. It should also be noted that analysis of aggrecan aggregates were made directly on the media samples. However, the graft extracts required 4M Guanidine HCl extraction and dialysis prior to CL-2B chromatography and this processing may have affected aggregation in spite of there being protease inhibitors present.

In summary, the results in this chapter have shown:-

- When *in vitro* cartilage grafts are generated in the presence of NIV or with NIV plus either selenium source there is a decreased sGAG and collagen biosynthesis suggesting NIV is the major contributor to cartilage pathology in this *in vitro* system that mimic KBD.

- When grafts are generated in the presence of either source of selenium (i.e. Na-Sel or Sel-Met) there are no major effects on sGAG production, however high doses of Na-Sel did show a significant increase in collagen production with similar non-significant trends in lower doses.

- Collectively, these results suggest that the deleterious effects of NIV exposure are independent from selenium supplementation.
- In all experiments exposure to selenium did not reverse the effects of nivalenol indicating that these two substances had independent effects on cartilage/ chondrocyte metabolism.

CHAPTER 5

DISCUSSION

Kashin-Beck disease (KBD) is endemic chronic degenerative osteoarthropathy whose main pathological changes occur in the growth plate and articular cartilage of human limbs and joints, where it is manifest as cartilage degeneration and necrosis. The effects of the disease on the growth plate therefore alter growth in children and can lead to growth arrest (short stature and limbs) and developmental deformities (crooked joints). In adulthood patients present with pain and deformity commonly affecting the hand, wrist, elbow, knee and ankle with radiological features and joint pattern involvement similar to those seen in rheumatoid arthritis (Farooq, Xiong et al. 2010). Past and current research suggests that KBD, with its endemic geographical distribution in China and Tibet, is due to the combined presence of fungal mycotoxins (found on the stored food ingested by affected populations) and a regional selenium deficiency in the environment providing local food sources. This evidence is supported by the correlation between the geographical occurrence of these 2 factors and the incidence of KBD. Fungal mycotoxins that have been implicated are nivalenol, butenolide and T-2 toxin. However, the cause of KBD still remains unclear as these 2 factors alone cannot explain all cases of KBD; many other villages in China also have mycotoxin contaminated water/ food sources and selenium deficiency but are KBD free. Mo et al, 1997 showed that selenium deficiency does not cause KBD but is closely related. Other causes that have been postulated are iodine deficiency, parvovirus B19, organic matter (fulvic acid) in drinking water and genetic factors.

At present there remains no preventative treatment for KBD and patients can only be treated for their symptoms of pain and correction of deformity. Recent randomised studies have shown improved pain scores with hyaluronic acid (intra-articular injection) and oral meloxicam (Tang, Pei et al. 2012) and chondroitin sulphate and/or glucosamine hydrochloride (Yue, Yang et al. 2012). Further understanding of the gene networks and signalling pathways involved in the pathogenesis of KBD and other chondral diseases may represent the future treatment of these conditions (Wang, Guo et al. 2012).

The Aims of this study were to examine the effects of a mycotoxin i.e. Nivalenol (NIV) and two different selenium sources on *in vitro* chondrocyte culture systems to better understand the cellular and molecular mechanism underlying the pathogenesis of KBD.

The two different sources of selenium used were sodium selenite (Na-Sel, chemical formula Na_2O_3Se) and selenium-l-methionine (Sel-Met, chemical formula $C_5H_{11}NO_2Se$). No previous studies have examined possible differences between these different selenium sources in cartilage development, or in the pathogenesis of KBD. It is not clearly understood which form of selenium is present in food sources or indeed if it is further metabolised into other forms after consumption. A recent review of the role of selenium in human health and disease concluded that there are significant knowledge gaps in the understanding of selenium metabolism and the requirements for optimal health (Fairweather-Tait, Bao et al. 2011). The majority of studies have tended to examine the effects of sodium selenite. A systemic review of randomised trials assessing the effectiveness of sodium selenite for the treatment of KBD in children suggested that sodium selenite is more effective than no treatment or placebo in patients with KBD, however it did recognise potential study biases and the need for further high quality, large number randomised controlled trials (Jirong, Huiyun et al. 2012). In our study there were no huge differences seen when comparing the 2 different selenium sources, however some of the results inferred a slight improvement with sodium selenite.

Interest in KBD has increased in the past few years, reflected in the increased number of published studies on the subject. These studies are not purely science based, but also examine current treatment options as well as developing a validated disease-specific assessment of quality of life measurement; KBDQOL (Fang, Guo et al. 2012).

Recent studies from our group have shown that altered pro-inflammatory cytokine metabolism (CD44, IL-1α and TNF-α) occurs in the pathogenesis of KBD, resulting in aggrecanase-generated proteoglycan loss in articular cartilage (Cao, Li et al. 2008). Another study has shown that cartilage neo-grafts cultured in the presence of fungal mycotoxins (including nivalenol) resulted in the loss of aggrecan and type II collagen, but increased type X collagen expression, supporting the hypothesis that mycotoxins are implicated as causative factors for KBD (Lu, Cao et al. 2012). Another collaboration between our group and the Xi'an Jiaotong University, China showed similar effects to that of nivalenol with T-2 toxin, which inhibited aggrecan synthesis, promoted aggrecanases and pro-inflammatory cytokine production, and consequently induced aggrecan degradation in chondrocytes. Some partial inhibition of these effects was seen with addition of selenium (Li, Cao et al. 2008).

Comparison of gene pathways of osteoarthritis (OA) and KBD has shown that reactive oxygen species are involved in the pathogenesis of OA with nitric oxide (NO) mediated chondrocyte apoptosis contributing to the development of KBD (Zhang, Guo et al.

2012). Another study implicated reactive oxygen species in the effects of T-2 toxin on chondrocytes in the development of disease processes in KBD (Tian, Wang et al. 2011).

Contributions and Conclusions from this study

The objective of this study was to develop an *in vitro* cartilage culture system to mimic the changes seen in KBD and thus determine the effects different sources of selenium, in the presence or absence of Nivalenol, had on cartilage neograft metabolism. Our hypothesis was that growth and metabolism of cartilage will be affected by exposure to either selenium or the mycotoxin Nivalenol or both in combination and these effects will mimic those found in KBD.

In Chapter 3 we investigated the use of chondrocytes grown in Transwell cultures as a method to mimic potential environmental conditions that could affect cartilage development, growth and function in KBD areas. Chondrocytes were isolated from 7 day old bovine articular cartilage following collagenase and pronase digestion and seeded at 4 x 10^6 cells into Millipore filter Transwells within 24 well plates. Media was changed thrice weekly and stored for analysis. The culture period was 28 days. At the termination of culture the media from the Transwell was removed/ harvested and the resulting cartilage graft was weighed and then analysed. An overview of the methodology was shown in Figure 4.1.

Initially we did not know appropriate doses of the compounds to use as there were no previous similar studies to reference from. The doses used of sodium selenite (Na-Sel) and Nivalenol (NIV) were 0.1, 0.5 and 1µg/ml, and in general it was found that both Na-Sel and NIV had a dose dependant negative effect on histological organisation, graft weight, graft thickness, cellular metabolism (lactate) and sGAG production. In view of these findings the doses were reduced by a factor of 10 for subsequent experiments. With the new doses cellular metabolism was found to be fairly uniform for all treatments. Cell viability was assessed using the MTT assay to ensure grossly toxic effects were not occurring. Negative effects were seen in the Nivalenol groups in the latter culture stages with some potential recovery seen when combined with the selenium compounds.

Assessment of graft weight and histology demonstrated that Nivalenol had negative effects on cartilage graft development at all doses, but a dose dependant pattern was seen. Using the adjusted doses of the selenium compounds, no significant differences were seen

with regards histological appearance and graft weight when compared to the control group. When selenium and Nivalenol were combined results were similar to those seen with Nivalenol alone, although a slight increase was seen in terms of graft weight when sodium selenite was added to Nivalenol. To my knowledge these findings are the first reported of this phenomenon, with no other studies examining effects of these compounds *in vitro* using a Transwell cartilage culture system.

In Chapter 4 I undertook a more detailed analysis of the graft cartilage matrix composition. Production, in the presence of the selenium compounds, Nivalenol and in combination, of sGAG and collagen in the culture media and the graft itself were analysed, as was aggrecan aggregation. The general findings of these analyses were that cartilage grafts generated in the presence of Nivalenol (either alone or in combination with either selenium compound) synthesised decreased levels of sGAG and collagen. In comparison the cartilage grafts generated in the presence of either selenium compound showed no significant differences to the control group with regards sGAG production, but there was an increase in collagen production seen with sodium selenite. In all experiments exposure to selenium did not reverse the effects of nivalenol indicating that these two substances had independent effects on cartilage/ chondrocyte metabolism.

Collectively, these results suggest that Nivalenol is the major contributor to cartilage pathology in this *in vitro* system that mimics KBD, and that these deleterious effects are largely independent from selenium supplementation. Again, to my knowledge these are the first described findings of their type.

It is difficult to assess whether this *in vitro* system is a true reflection of the pathological processes seen in KBD *in vivo.* It is still not known what the actual cause of KBD is, and it may well be multi-factorial. Certainly there is an argument to say that this study over-simplifies the role of Nivalenol and in particular selenium by implying direct exposure of these compounds can cause or protect against KBD development. There are probably complex pathways that are involved especially when one considers that these compounds are consumed in the diet or drinking water and would be subsequently metabolised by the small/large bowel, hepatic or renal systems.

What next?

With so many postulated causes of KBD (including the implication of other fungal mycotoxins) it is obvious that these could also be studied using a similar method to this study. The role of iodine deficiency would probably be very difficult to establish as it is highly likely that if this does indeed have a role in the pathogenesis in KBD it is via its effects on the hormone thyroxine. Our Transwell culture system would not be able to study the possible genetic causes. Undoubtedly, further analysis of the media and grafts produced in this culture system could be undertaken including;

Future work;

- More detailed analysis of the type of sGAG produced, differentiating between keratan and chondroitin sulphate using gel electrophoresis or ELISA assay techniques

- More detailed analysis of the collagen types produced

- Further immunohistochemistry analysis using the amended doses of Nivalenol and both selenium compounds

- Assessment using qPCR to investigate differences in mRNA expression of key enzymes involved in cartilage matrix biosynthesis and metabolism

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