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**The development of RNA extraction
protocols to examine the effects of early
exercise on gene expression in the
articular cartilage and subchondral
bone of Perendale sheep**

**A thesis presented in partial fulfilment of the
requirements for the degree of Master of Science**

at

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ABBREVIATIONS

AC	Articular cartilage
ADAM TS	Disintegrin matrix metalloproteinases with thrombospondin repeats
ADAMS	Disintegrin matrix metalloproteinases
BMD	Bone mineral density
BMP	Bone morphogenic protein
CT	Computed tomography
DJD	Degenerative joint disease
DXA	X-ray absorptiometry dual energy
GAPDH	Glyceraldehyde phosphate dehydrogenase
IL-1β	Interleukin-1-beta
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
OA	Osteoarthritis
OCD	Osteochondrosis dissecans
PCR	Polymerase chain reaction
PRF	Peptide regulatory factors
RA	Rheumatoid arthritis
RER	Rough endoplasmic reticulum
RT	Reverse transcription
Rt	Real time
SCB	Subchondral bone
TIMP	Tissue inhibitor metalloproteinase
TNF-α	Tumour necrosis factor alpha
US	Ultrasonography
VEGF	Vascular endothelial growth factors
β-Actin	Beta actin

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ABSTRACT

To examine gene expression *in vivo*, total RNA was extracted from articular cartilage and subchondral bone. As limited methodology existed for ovine, RNA extraction was performed by optimization of previously published protocols used for other species and *in vitro* studies (Chomczynski & Mackey, 1995; Chomczynski & Sacchi, 1987; Heinrichs et al., 1997). Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify the extracted RNA to evaluate the gene expression of inflammatory cytokines, collagens, collagenase and housekeeping genes glyceraldehyde phosphate dehydrogenase and Beta-Actin. We observed changes in the expression of inflammatory cytokines, collagen and collagenase genes between exercised and unexercised sheep. The results of this research are consistent with clinical imaging and microscopy studies which suggest that moderate exercise during early life can stimulate an adaptive response in articular cartilage and subchondral bone (Brama, Tekoppele, Bank, Barneveld, & van Weeren, 2000; Firth, 2006; Firth & Rogers, 2005b; Lammi et al., 1993). These changes can have a chondro-protective effect (Jones, Bennell, & Cicuttini, 2003; Otterness et al., 1998) and may reduce susceptibility to athletic injury in later life. Future research using fluorescent probes and polymerase chain reaction may permit quantification of gene expression in real-time to determine the anabolic and catabolic response of articular cartilage with developmental age and exercise *in vivo*.

Chapter 1

Introduction

1.1 Background

Osteoarthritis (OA) is the most common degenerative joint disease (DJD) affecting terrestrial vertebrates (Bhosale & Richards, 2008). It is a heterogeneous group of conditions that culminate in defective articular cartilage (AC) and bone loss. OA is characterized by slow and progressive cartilage deterioration, subchondral bone (SCB) remodeling, and inflammation of the synovium, pain and increasing disability (Pelletier, Martel-Pelletier, & Abramson, 2001; Schlueter & Orth, 2004). By 65 years of age more than 50% of people are afflicted with OA (M. B. Goldring, 2006). With an increasingly aging population, DJD is a growing epidemiological problem (Schaller et al., 2005).

Many mammals develop OA spontaneously (Huebner, Otterness, Freund, Caterson, & Kraus, 1998) and this causes significant financial and welfare cost to animal products (Arican, Coughlan, Clegg, & Carter, 2000; Hernandez & Hawkins, 2001). In sport and racing horses OA and lameness are the most important causes of poor performance and early retirement of the equine athlete (Jeffcott, Rosedale, Freestone, Frank, & Towers-Clark, 1982; Pool, 1996; Schlueter & Orth, 2004; R. J. Todhunter, 1996). The tarsus (hock) is the most commonly affected hind limb region associated with lameness in the horse (Vanderperren, Raes, Bree, & Saunders, 2009).

Injury as a result of cyclic trauma, normal and abnormal stressors, genetics and age can initiate degenerative changes within the synovial joint (McIlwraith, 1996). In horses, these changes can include inflammation of the synovium, degradation of AC and sclerosis of the underlying SCB (Cantley, Firth, Delahunt, Pfeiffer, & Thompson, 1999).

The resilience of AC to withstand mechanical strain is dependent on the structural organization of extracellular matrix (ECM) macromolecules and chondrocyte viability (Brama, Tekoppele, Bank, Barneveld, Firth, et al., 2000). Damage to the collagen network can cause chondrocyte injury, disrupt ECM metabolism and failure to maintain proteoglycan (PG) content. An increase in bone turnover and sclerosis of SCB are important features of DJD (Hayami et al., 2006). However, the involvement of inflammatory mediators originating in AC have been observed in the absence of SCB loss (McIlwraith, 1996).

Osteoarthritic lesions are often localized to weight bearing cartilage and sites of trauma. The local actions of interleukin-1 β (IL-1 β) and tumour-necrosis-factor- α (TNF- α) have been associated with cartilage degradation and the initiation of inflammatory processes. A catabolic response to injury outweighs synthetic processes and results in progressive ECM degeneration and cartilage loss (M. B. Goldring, 2000a).

Matrix metalloproteinases (MMPs) play an important role in the turnover of cartilage ECM under normal and pathological conditions (Nguyen, Murphy, Hughes, Mort, & Roughley, 1993). During growth and skeletal development, the degradative process is balanced by the expression of proteinases and their biological inhibitors (tissue inhibitor metalloproteinase, TIMP) (M. B. Goldring, 2000a). In normal adult tissue, MMP activity is very low and the ECM is in a state of dynamic equilibrium. This is in contrast to the upregulated/activated state of MMP's observed during physiological remodeling or inflammation (Johnson, Dyer, & Hupe, 1998). Proinflammatory cytokines, growth factors and ECM interactions (mechanical stress and pericellular environment) are key initiators in these processes.

Exercise is necessary for the maintenance of healthy AC (Otterness et al., 1998). Mechanical strain within physiological range has chondroprotective effects. Exercise of a moderate intensity and cyclic load rate cause an increase in PG synthesis and content, and increase in stiffness of the collagen fibrillar network. Joint disuse and exercise intensity that exceeds physiological constraint result in cartilage fibrillation, pitting, fissuring and gradual deterioration of the matrix and collagen network (Otterness et al., 1998).

An adaptive response to exercise has been observed in AC and SCB in various animal models including the horse, dog, cow, sheep, rabbit, rodents and non-human primates (Brandt, 2003). These studies identify areas of ongoing and future research regarding the pathology of joint disease. However, anatomical differences, lack of age matched controls, differences in the joint examined, individuals and exercise regime make it difficult to generalize data across species. Few studies have reported on the interrelationship between exercise, ageing and cartilage (Arokoski, Jurvelin, Vaatainen, & Helminen, 2000).

This research will be the first to examine gene expression in AC and SCB *in vivo* in exercised and unexercised pasture maintained sheep. This research will contribute to the limited knowledge of the signaling events that regulate the adaptation and growth of AC and SCB under the influence of exercise and developmental age.

1.2 Literature Review

1.2.1 Synovial joint structure & function

The joints of the vertebrate skeleton allow the connection of opposing bones, movement and mechanical support. Joint organs consist of bone, AC and soft periarticular tissues (R. J. Todhunter, 1996). Joint morphology determines structural and functional properties.

Three classifications of joints are observed, based on connective tissues and range of articulation. Synarthrodial joints are immovable, amphiarthrodial joints allow a small degree of movement, diarthrodial or synovial joints are freely moveable. Synovial joints are the most common and complex joints in the body and a variety of types exist.

The synovial joint (Figure 1.1) provides lubricated contact between the surface of two moving bones and functions to facilitate movement and reassign load between bones (R. J. Todhunter, 1996). Characteristic properties of the synovial joint include separation of the articulating bones by a joint cavity and a thin layer of hyaline cartilage enveloping the articulating surfaces. Near frictionless movement can be achieved by separation of the joint surfaces with synovial fluid.

The joint capsule and ligament tissue protect and stabilize the joint. The synovium provides nutrition and lubrication. Normal function is dependent on the integrity of the joint structures and the specialized properties of AC.

i) Joint capsule & ligaments

The articular capsule and ligaments govern range of motion, maintain joint function and prevent dislocation (Caron, 1999). The articular capsule consists of two separate layers: a thick outer *stratum fibrosum* and thin inner *stratum synoviale*. The *stratum synoviale* secretes and absorbs synovial fluid. Local thickening of the *stratum fibrosum* in certain areas form the ligaments which connect adjacent bones, stabilize the joint and can be intracapsular and or extracapsular.

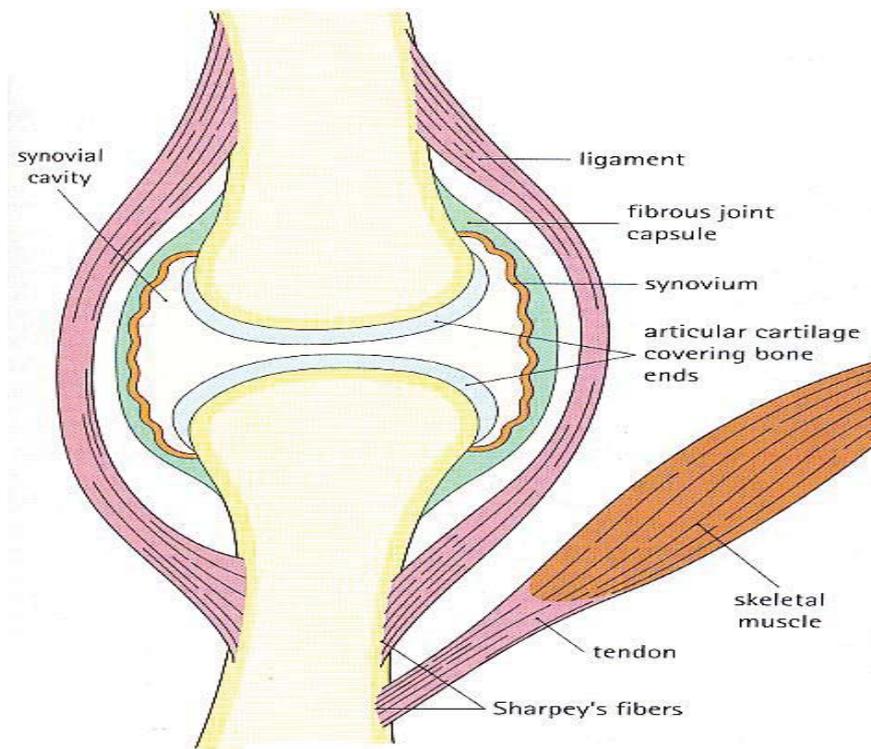


Figure 1.1 Diagrammatic representation of a synovial joint

The ends of the articulating bones are covered with articular cartilage and separated by the joint cavity. A fibrous capsule connects the two bones and is lined by synovium which secretes the synovial fluid that invests the joint and provides for lubrication.

Sourced: Stevens and Lowe, *Color Histology*, p. 249, Figure 13.28
www.mc.vanderbilt.edu/histology/labmanual2002...

Ligaments are composed predominantly of water (70%), collagen type I, elastin and small amounts of PGs and other proteins. The capsular and ligament tissues are innervated; receive blood supply and lymphatic drainage.

ii) Synovial membrane

The synovium is a thin tissue, which lines the joint space and defines the limits of the synovial cavity. The membrane originates from the AC boundaries of adjacent bones (Frandsen, Wilke, & Fails, 2003) and consists of synovial intima and subsynovial fibrous, areolar and fatty tissues which function to stabilize the joint, connect adjacent bones and increase surface area (*plicae synoviales*). Villi (*villi synoviales*) project into the joint cavity (Caron, 1999). During inflammatory conditions, the synovium contributes to joint degradation (Smith, 1999).

The synovium produces three specialized types of cells (synoviocytes) which produce a number of macromolecules (Caron, 1999). Type A cells are macrophagic and synthesize hyaluronan. Type B cells have secretory, fibroblastic and phagocytic ability and produce collagen, hyaluronic acid, proinflammatory mediators, cytokines, eicosanoids and proteases. Type C cells provide both functional and phagocytic properties and are considered a transitional cell type.

iii) Joint innervation & blood supply

The synovial joint is a highly innervated and vascularized organ equipped with proprio- and nociceptors (Bonnet & Walsh, 2005; Dowd, McQueen, Chessell, & Humphrey, 1998). Afferent output from mechanoreceptors within the joint can alert the central nervous system of impending injury which may be avoided through reflex systems. Innervation of the synovium may stimulate an inflammatory response to pain and protect the joint from injury or disease (Mapp, 1995).

iv) Synovial fluid & lubrication

Articular cartilage is without vascular, neural or lymphatic supply and dependent on nutrient diffusion from surrounding tissues (synovial fluid in mature subjects as well as SCB in immature subjects) (Maroudas, Bullough,

Swanson, & Freeman, 1968). According to Ficks Law, the movement of a solute into a membrane is dependent on the concentration gradient of the solute across the membrane and the diffusion coefficient. As the permeability of a solute is increased by agitation, joint movement and weight bearing play an important role in the nutrition of AC (Maroudas et al., 1968).

Hyaluronic acid (HA) is a major component of synovial fluid (C.W Archer, Dowthwaite, & Francis-West, 2003). HA and other low molecular weight proteins provide viscosity, soft tissue lubrication and shock absorption (Caron, 1999; Moreland, 2003). HA covers the surface of AC, is between collagen fibrils and sulfated PGs and protects chondrocytes and the collagen network from mechanical stress and deformation (Balazs, 1974). HA may protect the cartilage matrix from PG loss into the synovial space as well as prevent the invasion of inflammatory cells (Moreland, 2003).

The lubricating and deformable properties of articular surfaces are due to their separation by synovial fluid under hydrodynamic and elastohydrodynamic pressure and boundary lubrication (Wright & Dowson, 1976). Normal surface motion draws in the entrapped synovial fluid film and gives rise to a squeeze film or hydrostatic lubrication (Figure 1.2). Damage to the articular surface through mechanical or biochemical means, disrupts tissue integrity and lead to loss of fluid pressurization and increased stress on the collagen-PG network (Ateshian & Wang, 1995).

v) *The extracellular matrix*

The connective tissues of the joint capsule, ligaments, and menisci are composed of collagen, PGs, non-collagenous proteins and water. Collagen type I predominates in these connective tissues with smaller amounts of collagen types III and V.

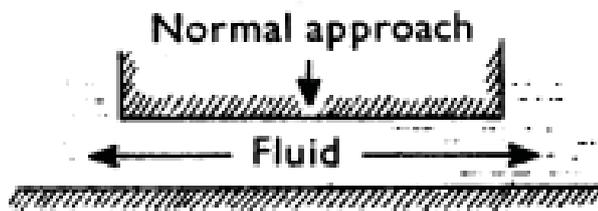


Figure 1.2 Squeeze film lubrication between two loaded surfaces

Both surfaces are completely separated by a fluid film i.e. synovial fluid. Under hydrodynamic pressure, surface motion draws fluid between the two surfaces. Articular cartilage deforms under hydrodynamic pressure and elastohydrodynamic lubrication. When the two surfaces approach each other in a normal direction the entrapped fluid gives rise to squeeze film lubrication.

Sourced: (Wright & Dowson, 1976) adapted.

1.2.2 Osteochondral tissues

The osteochondral tissues of the synovial joint include AC and subchondral mineralized tissues: calcified cartilage, subchondral cortical bone and subchondral trabecular bone (Figure 1.3) (Burr, 2004). Collectively, these structures support the joint surface and function (Holopainen et al., 2008; R. J. Todhunter, 1996).

The most superficial tissue is AC; a viscoelastic connective tissue designed to absorb shock and sustain normal joint function. Beneath the AC and separated from it by the tidemark is the calcified cartilage, an intermediary zone between the compliant AC and stiffer underlying SCB designed to reduce shear stress (Mente & Lewis, 1994). Unlike AC, calcified cartilage undergoes continued endochondral ossification throughout life (Burr, 2004). The junction between calcified and non-calcified cartilage becomes thin with aging and injury evident by reduplication of the tidemark (Burr, 2004; Secombe, Firth, Perkins, & Anderson, 2002). Deep to the calcified cartilage is the cortical SCB plate which demarcates the trabecular bone. SCB provides structural support to the superficial cartilage layers.

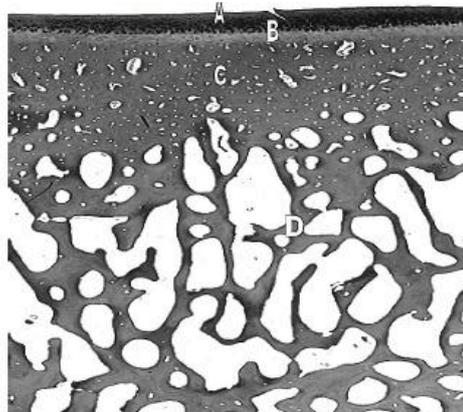


Figure 1.3 The osteochondral tissues

A histological section of the osteochondral tissues of the articular joint. (A) articular cartilage, (B) calcified cartilage, (C) subchondral bone plate, (D) trabecular bone. Sourced: Kawcak et. al. (2001).

i) Articular cartilage

Articular cartilage is derived from the hyaline cartilage template formed during embryogenesis. This specialized connective tissue persists throughout adult life and is equipped with unique hydrodynamic properties. During development, the mechanical environment determines cartilage surface geometry, contour and topological variation in thickness (Wong & Carter, 2003). Collagen fibrils and PG aggregates enable a low-friction gliding surface and smooth articulation of the joint (M. B. Goldring, 2000a; Hamerman & Schubert, 1962; McIlwraith, 1996; C. A. Poole, 2000; Stockwell, 1991).

Articular cartilage covers the ends of long bones and protects underlying subchondral mineralized tissues from peak mechanical, shearing and compressive forces generated during locomotion and weight bearing (Kawcak, McIlwraith, Nordin, Park, & James, 2001; Stockwell, 1991). The physical properties of AC are dependent on the organization and interaction of matrix macromolecules: water, collagen PGs, and non-collagenous glycoproteins (Johnston, 1997; C. A. Poole, 2000; Smith, 1999).

Articular cartilage is divided into four horizontal zones distinguished by chondrocyte morphology and orientation of collagen type II fibres (Figure 1.4)

(Clarke, 1971; McIlwraith, 2005; Torzilli, Deng, & Ramcharan, 2006; van Turnhout et al., 2008). Fibre orientation varies with tissue depth, age, species and adaptation to withstand derangement (C.W Archer et al., 2003; Clarke, 1971). AC thickness is greatest across convex surfaces, less on concave surfaces and decreases in thickness with age (Serafini-Fracassini & Smith, 1974; Wong & Carter, 2003).

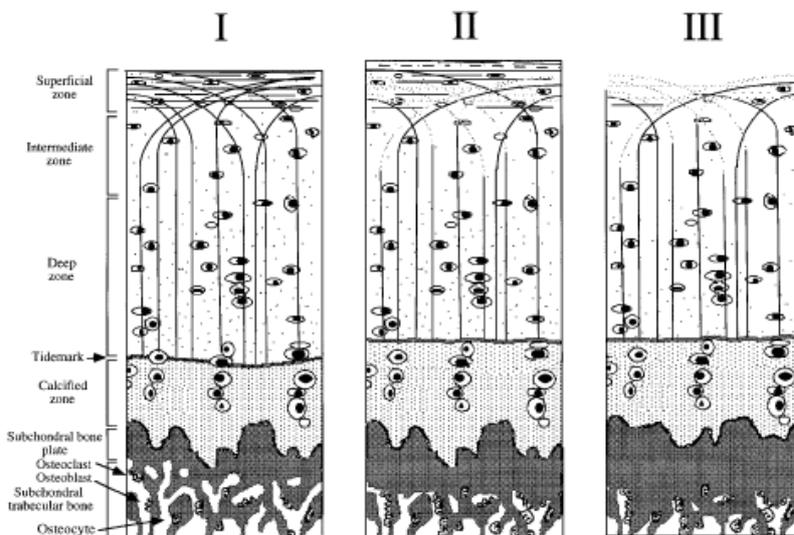


Figure 1.4 Collagen fibre orientation and the etiopathogenesis of osteoarthritis in the Beagle dog.

Oval structures represent chondrocytes, dots represent glycosaminoglycans (GAGs) and curved lines represent collagen fibrils. (I) Normal histology: articular surface is intact and collagen fibrils are tightly bound and orientated parallel with, and perpendicular to, the surface extending from superficial to deep zones. A high content of GAGs is evident. (II) Disease onset: a loss of GAG and exposure of superficial collagen fibrils results in disorganization of the collagen network. An increase in water content, thickening and softening of the cartilage layer ensues. In addition, thickening of calcified cartilage and subchondral bone plate occurs with increased trabecular bone remodeling. (III) With disease progression, increased GAG content loss, advancing tidemark and subchondral bone loss. Pathology is irreversible. Sourced: Arosoki (2000) adapted.

a) Chondrocytes

Chondrocytes are the singular cells of AC and comprise 1-12% of the ECM (M. B. Goldring, 2000b; McIlwraith, 1996). Chondrocytes are arranged in clusters or singly within spaces referred to as lacunae and regulate matrix metabolism in a highly organized and efficient manner (Stockwell, 1979a; Tyler, Bird, & Giller, 1990). Chondrocytes synthesize matrix components during growth and development and maintain tissue homeostasis during adult life responding to stimuli with increased activity (Hedbom & Hauselmann, 2002). Chondrocytes are embedded within the ECM and stabilized by the interactions within matrix macromolecules.

The pericellular environment immediately surrounding the chondrocytes is composed mainly of collagen type VI. This environment functions to support chondrocyte viability, providing structural, functional and metabolic stability (Bhosale & Richards, 2008; C. A. Poole, 2000). With increasing distance from cells, there is a decrease in matrix metabolism and distant interterritorial regions may be susceptible to deterioration, especially in mature adult tissue (Jadin et al., 2005). During growth and maturation a high density of cells are observed in the superficial zone.

Chondrocytes have high individual metabolic activity. However, AC is hypocellular and over matrix volume metabolic activity is low (Bhosale & Richards, 2008). Due to the low oxygen concentration of the matrix, chondrocytes are dependent on anaerobic metabolism (M. B. Goldring, 2006; Platt, 1996). As a result, cartilage matrix proteins have a low turnover rate in healthy adult tissue (Byers & Brown, 1990).

Chondrocytes influence the homeostatic balance of the ECM in response to mechanical stimuli, growth factors, and cytokines (M. B. Goldring, 2006; Platt, 1996). Regional differences in chondrocyte activity exist, with an increase in ECM remodeling in the immediate pericellular zones. Cartilage composition and function are intimately related (C. A. Poole, 2000) and disruption to chondrocyte morphology can alter collagen synthesis and initiate release and

activation of enzymes capable of ECM degradation (Smith, 1999; Woessner, 1991).

Chondrocytes have little ability to repair or reorganize the structural architecture of the matrix when it has reached its mature adult state (Barbero et al., 2004). Damage to collagen type II at the superficial layer can progress to mid and deeper zones with age and during OA (Hollander et al., 1995). Consequently, cartilage degeneration can lead to irreversible damage and DJD (O'Rear et al., 2005). During tissue turnover fragmented matrix macromolecules can be found in the synovial fluid (Saxne & Heinegard, 1995).

b) The extracellular matrix

The extracellular matrix consists of collagen fibrils, PGs, water and chondrocytes. The collagen network gives the matrix tensile strength and integrity, PGs and high osmotic pressure maintain tissue hydration and chondrocytes maintain matrix metabolism. The ECM reflects the loading history of AC and functions to protect chondrocytes from mechanical load, store cytokines and growth factors, and nutrient diffusion to chondrocytes (Bhosale & Richards, 2008).

The matrix acts as a signal transducer and when it deforms under load, produces mechanical, electrical and chemical signals which affect chondrocyte function. The ability of AC to resist compressive, shear and tensile forces is dependent on the integrity of the ECM and chondrocyte morphology (Grodzinsky, Levenston, Moonsoo, & Frank, 2000). Any defect in matrix organization can result in structural malfunction and cartilage degradation (Figure 1.4) (Stockwell, 1991).

c) Water content

The water content of AC comprises 65-80% of wet weight (Bhosale & Richards, 2008) and is dependent on interaction with large hydrophilic negatively charged aggregating PGs (Smith, 1999). Water allows the deformation of the cartilage dependent on load and provides nutrition and medium for lubrication (Maroudas et al., 1968). Water content declines with age from 80-90% in fetal tissue to 70% in adult (Bhosale & Richards, 2008).

Following injury, water content is observed to increase due to increased permeability and disruption of the cartilage matrix.

d) Proteoglycans & aggrecan

Proteoglycans form 10-20% wet weight of the ECM and consist of one or more glycosaminoglycan (GAG) chains covalently attached to a protein core (aggrecan) (R. J. Todhunter, 1996). The GAG chains are linear polymers of repeating disaccharides and contain a hexosamine and carboxylate or sulfate ester, or both. Link protein stabilizes a non-covalent interaction between aggrecan and HA which maintain fluid and electrolyte balance within the ECM by a Donnan effect (Bhosale & Richards, 2008).

Proteoglycans are produced by chondrocytes and secreted into the matrix as large aggregating monomers (aggrecans) or small non-aggregating PGs (decorin, biglycan and fibromodulin) (Bhosale & Richards, 2008). PGs provide compressive strength and the ability to resist compression due to their high osmotic attraction of water and sodium. The stiff collagen network limits swelling tendency and induces tensile stress (Bank, Soudry, Maroudas, Mizrahi, & TeKoppele, 2000)

Aggrecans make up the greatest percentage of PGs in AC and provide resistance to proteolytic attack (Smith, 1999). Aggrecans contain chondroitin sulphate and in most species keratan sulphate (Figure 1.5). The non-aggregating PGs in AC are characterised by chondroitin sulphate-dermatan sulphate PGs or keratan sulphate PGs. The role of these small leucine-rich PGs (SLRPG) *in vivo* is not fully understood (R. J. Todhunter, 1996). *In vitro* studies suggest SLRPs interact with collagen and regulate fibril diameter and matrix assembly (Iozzo, 1999; Scott, 1988).

Hyaluronic acid is the major GAG of AC and differs from other PGs as it is not covalently bound to a core protein when synthesized and is not sulphated (C.W Archer et al., 2003). It is a high molecular weight protein composed of units of glucuronate linked to N-acetyl glucosamine. In AC, HA forms a 'backbone' to which PGs attach via a hyaluronan binding region stabilized by a link protein

(Figure 1.6). HA and PGs form macromolecular aggregates several million KDa in molecular weight. A number of non-collagenous proteins have been described in AC including matrix proteins and link protein (P. G. Todhunter et al., 1996; Upholt & Olsen, 1991).

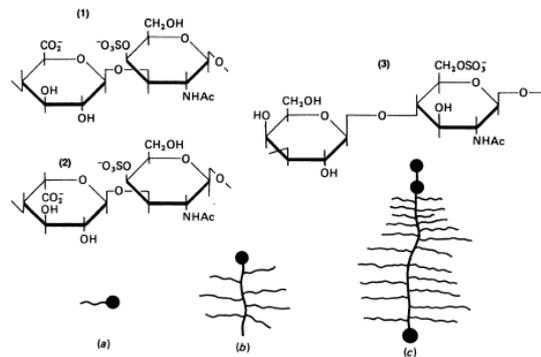


Figure 1.5 Proteoglycan: structure of repeating disaccharide units

The structures of repeating disaccharide units of glycan in proteoglycans which associate orthogonally along the collagen fibril at specific sites. Key: (1) chondroitin sulphate (as the 4-sulphate), (2) dermatan sulphate, (3) keratan sulphate (monosulphate). (a)-(c) diagrammatic representation of the (a) small (b) large (c) very large proteoglycans. Sourced: Moreland (2003) adapted.

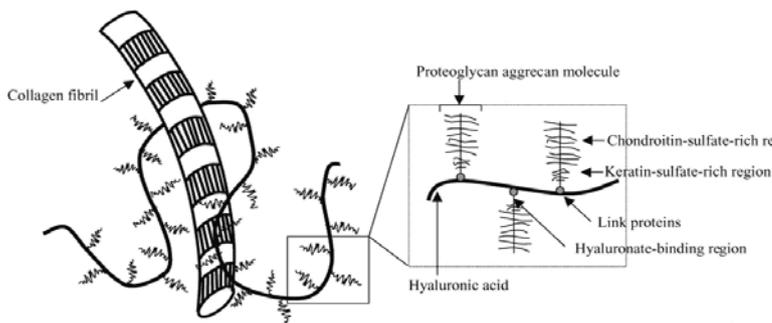


Figure 1.6 Proteoglycan attachment along collagen fibril

Proteoglycans are attached to a backbone of hyaluronic acid that is intertwined among collagen fibrils. Proteoglycans can have chondroitin-sulphate and keratan-sulphate rich regions. Link proteins assist the binding of aggrecan to hyaluronic acid. Sourced: Moreland (2003) adapted.

Proteoglycan turnover, unlike collagen, is rapid and percentage content within AC varies with age and tissue depth (Serafini-Fracassini & Smith, 1974). Fetal tissue contains little or no keratan sulphate. However, following birth the content of keratan sulphate increases in linear fashion throughout life. Similarly, chondroitin-6-sulphate remains constant during post natal life whilst the content of chondroitin-4-sulphate declines (Bailey & Mansell, 1997). GAG content in the superficial layers constitutes as little as 3% of dry weight and increases in content towards the cartilage-bone junction (Bailey & Mansell, 1997).

e) Articular cartilage collagen

Collagen type II and other minor collagen components (collagen types VI, IX, X, XI, XII, XIV) form a dense fibrillar network in which a high concentration of large chondroitin sulphate and keratan sulphate substituted PGs are embedded. The integrity of the collagen network determines AC stiffness and dynamic shear modulus as well as PG content (Oakley et al., 2004). Up to 95% of the collagen in AC is collagen type II which is most concentrated in the superficial zone

The tensile strength of AC can be attributed to the triple helix formation of collagen fibril polypeptide α -chains (Eyre, Wu, Niyibizi, & Chun, 1990) and cross-linking bonds formed with hydroxylysine-aldehyde residues (Eyre et al., 1990). Hydroxylysyl pyridinoline is the predominant cross-linking structure found in mature cartilage (Eyre et al., 1990). The cross-linking bonds between hydroxylysylpyridinoline in cartilage collagen types II, IX and XI are dominantly formed by an interaction between telopeptide domains of collagen type II (Eyre et al., 1990). Table 1.1 identifies the different collagen types in AC and their known functions and chain composition.

Table 1.1 Different collagen types in normal articular cartilage and their known functions.Sourced: *Bhosale and Richardson (2008), Todhunter (1996), Eyre (2001).*

Collagen Type	Chain Composition	Known Functions
II	$[\alpha_1(\text{II})]_3$	Most abundant (90-95%) collagen in hyaline cartilage forming the principal component of collagen macrofibrils providing tensile strength. Also found in developing cornea, vitreous humor
VI	$\alpha_1(\text{VI}) \alpha_2(\text{VI}) \alpha_3(\text{VI})$	Present in mammalian articular cartilage in the pericellular matrix but not avian. Forms microfibrils and may link larger collagen fibrils and chondrocyte attachment within matrix. Located in pericellular matrix and polymerizes to form filamentous network providing multiple adhesion domains for cells and matrix components
IX	$\alpha_1(\text{IX}) \alpha_2(\text{IX}) \alpha_3(\text{IX})$	Involved in fibril formation forming critical heteropolymer cross-linked with collagen II and XI. Located on surface of major collagen fibrils. Limits collagen fibril diameter and interacts with other matrix components. Provides tensile properties and cross-links to surface of macrofibrils. Mutations of collagen IX gene result in chondrodysplasia precocious osteoarthritis.
X	$[\alpha_1(\text{XI})]_3$	Related to hypertrophied cells during development and in deep calcified cartilage layer in adult, interfaces articular cartilage with bone. Provides structural support associated with mineralization.
XI	$\alpha_1(\text{XI}) \alpha_2(\text{XI}) \alpha_3(\text{XI})$	Located within or on macrofibrils of type II collagen. Believed to be involved in fibril formation forming critical heteropolymer cross-linked with collagen II and IX. Mutations of collagen XI gene result in chondrodysplasia precocious osteoarthritis.
XII	$[\alpha_1(\text{XII})]_3$	A minor collagen present in mammalian articular cartilage and connective tissues. Bound to fibril surfaces but not covalently attached
XIV	$[\alpha_1(\text{XIV})]_3$	Function not well understood. Structurally related to type IX collagen. Bound to fibril surfaces but not covalently attached

ii) Subchondral bone

Long bones originate from a hyaline cartilage template, rich in collagen type II and PGs, by endochondral ossification during the fetal stage of development. With formation, collagen type II is progressively replaced with fibrillar collagen type I essential for the deposition of bone mineral (C.W Archer, Morrison, Bayliss, & Ferguson, 1996).

Bone is composed of organic (30%) and inorganic (70%) compounds. Organic bone is made up of collagen (95%) and non-collagenous proteins (5%) including osteocalcin and bone sialoprotein. Mineral hydroxyapatite comprises the inorganic component and includes mainly calcium and phosphate. Collectively collagen type I and mineral density govern the biomechanical properties and functional integrity of bone. Collagen fibres contribute to bone structure, elasticity and ability to absorb energy. The mineral phase provides strength and stiffness.

Bone is an anisotropic and elastic material which continually adapts to physiological and mechanical stimuli through modeling and remodeling processes (Kawcak et al., 2001). Bone modeling, proposed by Wolff in 1892, is based on mathematical theory governed by mechanical and biological signals at the organ (macromodeling) or trabecular level (micromodeling) (Coelho, Fernandes, Rodrigues, Cardoso, & Guedes, 2009). Bone growth, modeling and remodeling are continuous throughout life and active to different degrees in different mineralized tissues (Burr, 2004).

Subchondral bone remodels rapidly and can change the shape and congruity of the joint. Fatigue and micro-damage can initiate this process and result in normal remodeling, excessive remodeling and sclerosis, or accumulation of micro-damage. This can lead to fracture or traumatic osteochondritis dissecans lesions (Schlueter & Orth, 2004).

Bone adapts in order to satisfy functional demand and metabolic cost (Coelho et al., 2009). During bone growth, formation results in increased bone mass. During bone modeling, the process of activation-formation or activation-resorption results in a net increase in mass and change in structural geometry. During bone remodeling activation-resorption-formation are coupled and contribute to bone maintenance (Burr, 2004). When mechanical stimulus is low, bone remodeling removes bone. When mechanical stimulus is excessively high, remodeling adds bone. Bone remodeling is kept at a relatively low level due to inhibitory signals produced through physiologic loading (R. B. Martin, 2000). In the normal joint, endochondral ossification and subchondral remodeling are in balance (Burr, 2004).

Subchondral bone acts as a shock absorber between AC and epiphyseal bone (Firth & Rogers, 2005b; Viguet-Carrin, Garnero, & Delmas, 2006) and maintains joint shape (Kawcak et al., 2001). SCB absorbs ground impact forces transmitted through the limb during locomotion and minimizes pathology at the articular surface (Secombe et al., 2002). The functional adaptive response of SCB, reflects the loading history of the articular surface (Eckstein, Muller-Gerbl, Steinlechner, Kierse, & Putz, 1995).

In horses, the basic collagen fibril network of SCB is established by 5 months of age (P.A.J. Brama, J.M. Tekoppele, R.A. Bank, A. Barneveld, & P.R. van Weeren, 2002; Holopainen et al., 2008). SCB mineral content and volume increase significantly with growth and maturation (Holopainen et al., 2008). Variation in SCB thickness can be observed across the joint surface consistent with the mechanical properties and imposed stressors at each site (Kawcak et al., 2001).

The ability of bone to resist fracture is dependent on bone quantity and quality and can be described in terms of architecture, turnover and mineral and organic properties of the matrix (Viguet-Carrin et al., 2006). During remodeling activation, resorption and formation can take days to weeks to complete. Following formation, 65-70% of new bone is quickly mineralized but total mineralization may take 12 months (Burr & Schaffler, 1997). Bone mineral

mass density has a strong inverse correlation with fracture risk (Lepage, Carstanjen, & Uebelhart, 2001).

Extensive bone remodeling may underlie overall SCB bone loss (Arokoski et al., 2000; Burr & Schaffler, 1997). The increased rate of bone turnover may result in newly formed bone not being well mineralized and overall material stiffness and elastic modulus reduced despite an increase in structural stiffness. Increased density in calcified cartilage in proximity to the tidemark may increase stress in the deep AC layer and contribute to cartilage loss. An increase in calcified cartilage mineral content and thickness with age and OA likely has greater effect on cartilage loss than does SCB (Burr, 2004).

Endochondral ossification and tidemark advancement occur throughout life and contribute to thickening of calcified cartilage depending on the modeling processes in SCB (Schlueter & Orth, 2004). However, remodeling at the osteochondral junction can cause calcified cartilage thinning. In normal joints remodeling and endochondral ossification are in balance.

1.2.3 The ovine hock joint

Cloven hoofed ungulates walk on their third and fourth digits and are in the order Artiodactyl. The sheep is a domestic ungulate in the suborder Ruminantia which includes the cow (*Bos Taurus*, *Bos indicus*), sheep (*Ovis aries*) and goat (*Capra hircus*).

The horse (*Equus caballus*) is an odd-toed ungulate of the Perissodactyla, suborder Hippomorpha and Equidae family. Equids characteristically have limb muscle close to the trunk with tendons extending over the long third metacarpal and metatarsal bones to the digits. This provides significant leverage and the ability to maintain rapid locomotion.

The skeleton of terrestrial vertebrates is designed to withstand and transmit loads experienced during locomotion. Maximum strain rate can be observed to increase linearly with speed but peak strain is dependent on gait. The

relationship between peak functional strain and failure of cortical bone in the radius and tibia of quadrupeds during vigorous activity is uniform (C. T. Rubin & Lanyon, 1982).

In quadrupeds the distal tibia and tarsal bones of the hind limb collectively form the hock joint (articulations pedis) and correspond to the human ankle. The hock joint is a complex series of joints formed of the tibio-tarsal, intertarsal, and tarso-metatarsal articulations (Figure 1.7). Differences between the talus of the sheep and horse exist.

In sheep, the bones which collectively comprise the hock joint include the: (i) lateral malleolus (distal end of fibula), (ii) calcaneus, (iii) talus (iv) fused central and fourth tarsal, (v) first tarsal, (vi) fused second and third tarsal bones. The weight of the hind limb is carried by the tibia.

In horses, the bones which collectively comprise the hock joint include: (i) lateral malleolus (distal end of fibula), (ii) calcaneus, (iii) talus, (iv) central tarsal bone, (v) fourth tarsal bone, (vi) fused third tarsal bone. The tarsus is the most commonly affected site of lameness in the hindlimb in horses (Vanderperren et al., 2009).

Due to the anatomical differences between the equine and ovine hock joint, articulation between the tibial cochlea and the trochlea of the talus are directed laterally in the horse where the hindfoot moves outside the forefoot when the horse gallops. In the sheep the talocalcral articulation is almost vertical (Skerritt & McLelland, 1984). In the horse the proximal intertarsal joint is formed between the talus and calcaneus proximally and the central and fourth tarsals distally. In contrast, that of the sheep articulates with the central tarsal bone via the trochlea (Skerritt & McLelland, 1984). The lateral malleolus is not fused and articulates with the tibia and the tibial and fibular tarsal bones (Figure 1.8).

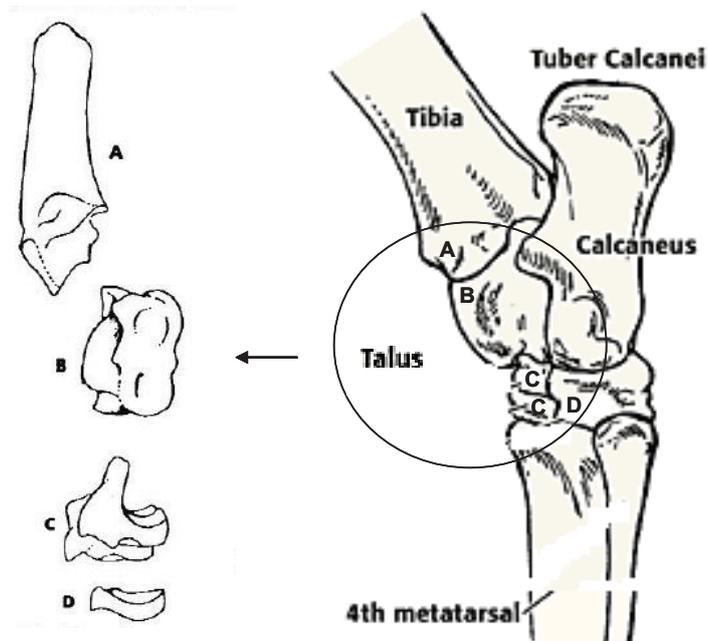


Figure 1.7 Lateral view of the hock joint.

In quadrupeds the distal tibia and bones of the hind limb collectively form the hock joint. The hock joint is a complex series of joints formed of the tibio-tarsal, intertarsal, and tarso-metatarsal articulations. The weight of the hind limb is carried by the tibia. The distal tibia articulates with the bones of the talus, see insert. Insert: Bones of the right talus: (A) Fibular tarsal; (B) Tibial tarsal; (C) Central-fourth tarsal; (D) Second-third tarsal. Sourced: www.thehorse.com May (1995) adapted.

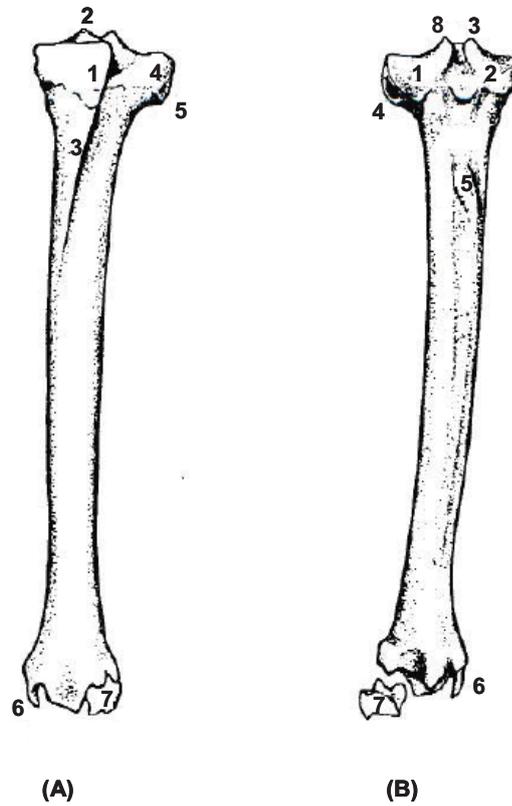


Figure 1.8 The left distal aspect of the tibia.

(A) Left distal tibia (dorsal view), (B) Left distal tibia (caudal view). 1. Tibial tuberosity; 2. Spines; 3. Tibial crest; 4. Lateral condyle; 5. Proximal end of fibula; 6. Medial malleolus; 7. Lateral malleolus; 8. Intercondyleoid fossa. Sourced: www.thehorse.com May (1995) adapted.

1.2.4 Joint growth, modeling & remodeling

Vertebrate long bones are formed by a highly coordinated and complex process of endochondral ossification (Provot & Schipani, 2005). Systemic and local factors regulate the gradual replacement of the embryonic cartilage skeleton with bone through intracellular signaling, and feedback systems (Malemud, 2006b). Systemic factors regulating chondrocyte behaviour include growth hormone and thyroid hormone. Intrinsic factors regulating chondrocyte gene expression and differentiation include: Sox proteins, fibroblast growth factors, bone morphogenic proteins (BMPs), parathyroid hormone and related peptides, Indian hedgehog and runt transcription factors, hypoxia-inducible factors and vascular endothelial growth factors (VEGF) (Table 1.2) (Figure 1.10) (Mackie, Ahmed, Tatarczuch, Chen, & Mirams, 2008; Provot & Schipani, 2005).

Synovial joints arise in long bone as a consequence of cartilaginous differentiation at the prospective joint site (C.W Archer et al., 2003). Key initiators are the production of hyaluronan by cells of the interzone and presumptive synovial cells and mechanical stimulus (C.W Archer et al., 2003; C.W. Archer, Morrison, & Pitsillides, 1994). Limb movement is necessary for joint cavitation and AC development (Arokoski et al., 2000). Growth differentiating factor-5, BMPs, BMP antagonist noggin, and Wnt-14 are also involved (Table 1.2) (C.W Archer et al., 2003). AC development is driven by appositional growth of progenitor/stem cell populations within the articular surface. AC has escaped endochondral ossification and is permanent at each end of the long bone.

Matrix metalloproteinases (MMPs) are critical factors involved in long bone growth, maturation and joint formation. MMPs induce cartilage growth plate angiogenesis and apoptosis of hypertrophic chondrocytes in conjunction with VEGF (Malemud, 2006b). The expression of MMPs 1, -2, -3, -9, -13, and -14 are temporally and spatially related to the stage of limb development in the rat (Figure 1.9) (Malemud, 2006b).

Table 1.2 Molecular mechanisms regulating endochondral bone development.

Sourced: Archer et al., (2003), Mackle et al., (2008), Malemud, (2005b), Provot & Schipani, (2005).

Early chondrogenesis in the limb	Actions:
Sox proteins: Sox9, L-Sox5, Sox6	Chondrocyte differentiation, proliferation & hypertrophy. Regulate expression of cartilage matrix proteins: collagen types II, IX, XI and aggrecan
Inhibitor of growth & chondrocyte proliferation Fibroblast growth factor (FGF) receptor3: FGF1, -2, -4, -8, -9, -18	FGFR3 expressed in round & columnar proliferating chondrocytes. FGF signaling hastens late stage chondrocyte hypertrophy. Mutation in FGFR3 gene results in human skeletal dysplasias including achondroplasia
Endochondral bone development Bone morphogenic proteins (BMP): BMP2, -3, -4, -5, -6, -7, Growth differentiation factor5, Noggin (BMP-inhibitor)	Growth & differentiation factors. Induce cartilage & bone formation. Crucial role in mesenchymal condensation and joint formation, induces apoptosis through the actions of Noggin. Noggin null mutations result in poor limb development & absence of peripheral phalangeal joints.
Mineral ion homeostasis & bone development: Parathyroid hormone-related peptide (PTHrP) & its receptor	PTHrP binds its receptor & is a key regulator of calcium/phosphate metabolism & bone remodeling. Found in fetal & adult: cartilage, kidney, heart, hair follicle, placenta, breast, lung, epithelial tissues. Functions as an autocrine, paracrine factor. Mutation or absence PTHrP/PTHrP receptor results in chondrodysplasias.; prenatal lethality, premature & abnormal bone mineralization & ossification, shortened limbs: Blomstrand's lethal chondrodysplasia, and Jansen's metaphyseal chondrodysplasia
Central coordinator of endochondral bone development: Indian hedgehog (Ihh), Sonic hedgehog (Shh), Smoothened (Smo), Patch (Ptc)	Ihh proteins expressed in the interzone between proliferating and hypertrophic chondrocytes, feedback loops with PTHrP, BMPs, FGFs & Runx2. Ihh inhibits hypertrophic chondrocyte differentiation & delays mineralization of cartilage matrix through negative feedback loop with PTHrP. Ihh is necessary for PTHrP expression. Ihh induces BMPs in a positive feedback loop. FGFs reduce Ihh expression. Runx2 upregulates Ihh expression & induces chondrocyte hypertrophy
Chondrocyte hypertrophy, Runt transcription factors: Runx2, Runx3	Runx2 essential to osteoblast differentiation. Runx2 expressed in chondrocytes & drives maturation & expression of hypertrophic markers & collagen X. Runx2 feedback activates Ihh & PTHrP expression which determines chondrocyte maturation & proliferation. Runx2 plays critical role in vascular invasion of cartilage through Runx2-dependent regulation of blood vessel invasion mediated by vascular endothelial growth factor.

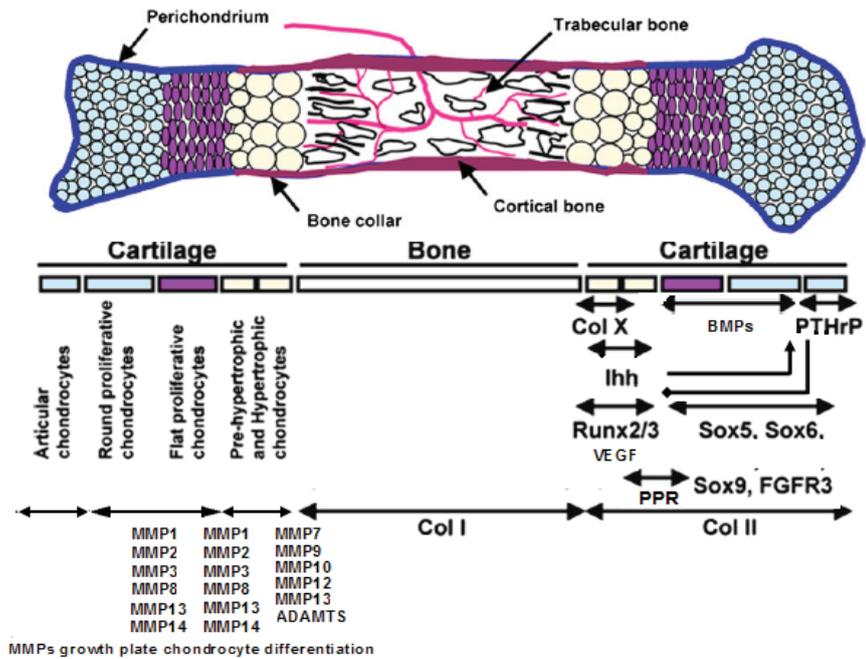


Figure 1.9 Endochondral bone formation

Schematic representation of a mouse tibia during late stage fetal development. The cartilaginous template is progressively calcified with the deposition of bone. Characteristic markers of bone formation, and chondrocyte differentiation are identified. Chondrocyte differentiation is under the regulation of MMPs, Sox proteins, fibroblast growth factor-3 (FGF3), Indian hedgehog (Ihh), Runx2/3, parathyroid hormone and related peptide (PTHrP) and its receptor (PPR). Collagen type I is characteristic of bone matrix, collagen types II and X are characteristic of articular cartilage. Sourced: Provot & Schipani (2005) adapted.

1.2.5 Degenerative joint disease: Osteoarthritis

The etiology of OA is multifactorial involving a complex interaction of inflammatory, metabolic and mechanical signals. Repetitive minor and acute trauma, immobilization, ligament or muscular instability, bony misalignment, joint sepsis, obesity, genetics and age can contribute to OA (Arokoski et al., 2000; Bhosale & Richards, 2008; Mcllwraith, 1996). In horses, high intensity training, biomechanics, farrier treatment, and environmental factors are also associated with DJD (Schlueter & Orth, 2004). It is widely held that once the integrity of AC in the superficial zone is compromised, the underlying cartilage is subjected to abnormally high strain (Arokoski et al., 2000) (Fig.1.10). Disease progression results in all tissues surrounding the joint becoming involved (Brandt, 2003; Oegema & Visco, 1999).

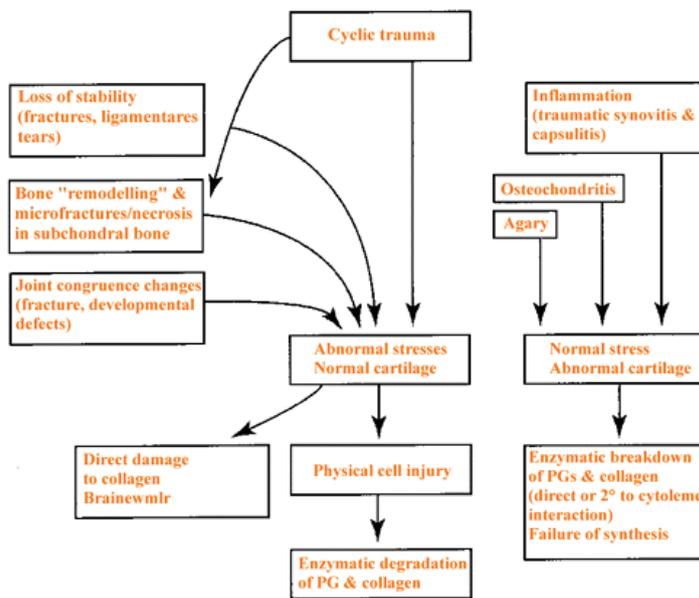


Figure 1.10 Factors involved in the initiation of cartilage degradation and onset of osteoarthritis

Sourced: Mcllwraith (1996) adapted.

The homeostatic balance in cartilage regulated by several factors produced by the synovium and chondrocytes including: cytokines, inflammatory mediators and MMPs (Brandt, 1997; M. B. Goldring, 2000b; Hollander et al., 1995; Johnston, 1997; Schlueter & Orth, 2004; Smith, 1999; R. J. Todhunter, 1996) (Figure 1.11). In normal cartilage, the balance between matrix synthesis and degradation is maintained. In OA, disruption to cartilage homeostasis and PG synthesis occurs. Disorganization of the type II collagen network and cartilage softening are early indicators of pathology (Hollander et al., 1995).

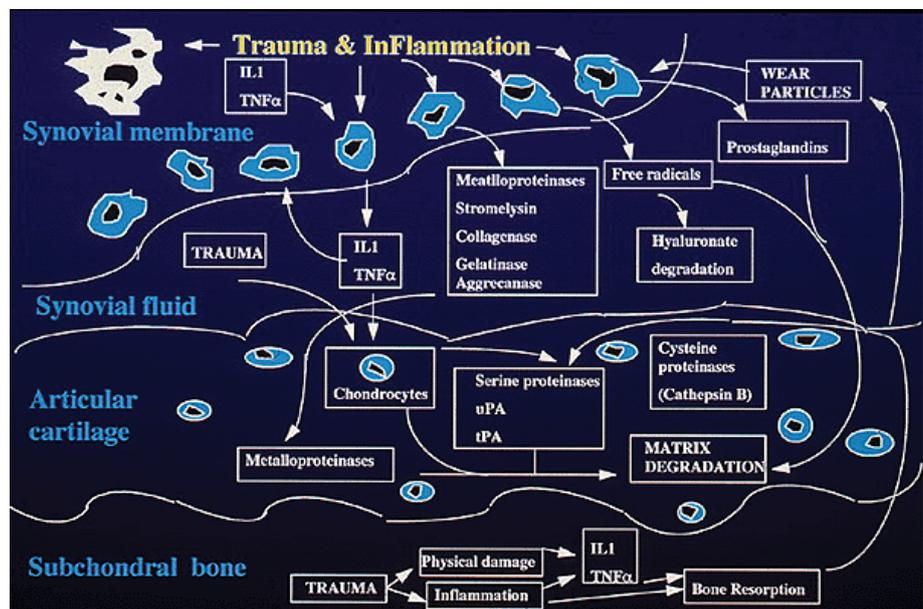


Figure 1.11 Enzymatic factors involved in cartilage degradation and onset of osteoarthritis

Sourced: McIlwraith (1995).

Defects in AC can arise secondary to either trauma or osteochondritis dissecans (OCD) and include partial and full thickness chondral and osteochondral lesions. Depth and size of the defect affect the repair process (Bhosale & Richards, 2008; Mankin, 1982). Partial thickness defects in adult AC do not heal spontaneously and enlarge over time. However, full chondral defects may heal transiently (Hunziker, 1999). A feature of the repair response

is renewed cell division and upregulated matrix synthesis which resemble some aspects of development (C.W. Archer et al., 1994).

Cartilage lesions are not characterised by pain unless they extend into SCB or are accompanied by synovitis (McIlwraith, 2005). Exposure of bone marrow mesenchymal progenitor cells and growth factors, initiate an intrinsic repair response (Redman, Oldfield, & Archer, 2005). However, the fibrocartilage repair tissue is inferior to hyaline cartilage and biomechanically cannot endure repetitive loading (Bhosale & Richards, 2008). A shift from collagen type I fibrous tissue synthesis to collagen type II articular cartilage synthesis within 5-6 weeks of subchondral injury has been observed (Bhosale & Richards, 2008).

Upregulation of collagen type II and aggrecan gene expression are observed in early OA, consistent with reported increases in cartilage thickness in the ovine OA model (Appleyard et al., 2003). However, the thicker cartilage is biomechanically inferior and undergoes degenerative change over time. This is a result of a loss and failure of articular chondrocytes to replace the water binding PGs. Chondrocytes are unable to maintain homeostatic balance and cartilage degradation exceeds synthesis (Stockwell, 1991). By the time clinical signs of disease are evident irreversible degenerative damage has occurred (Johnston, 1997).

Bone is intimately involved in the initiation and progression of OA (Anderson-MacKenzie et al., 2005; Burr, 2004; Fuller, Barr, Sharif, & Dieppe, 2001). However, changes in AC and SCB are closely related making it difficult to determine a single initiating factor in either tissue (Kawcak et al., 2001). This has led to disagreement as to whether changes in SCB precede, are simultaneous to, or follow changes in AC.

The process of endochondral ossification and subchondral remodeling are in a state of homeostasis in the normal joint. During OA, hypermineralisation in hyaline cartilage, calcified cartilage, and SCB has been reported in human femoral heads (Ferguson, Bushby, & Boyde, 2003). The highly mineralized fragments may contribute to wear of the articular surfaces, alter loading

patterns and contribute to joint pathology. Bovine *in vitro* studies have revealed that the extent of matrix damage and chondrocyte death are correlated to rate of mechanical load (Blain et al., 2001; Ewers, Dvoracek-Driksna, Orth, & Haut, 2001).

1.2.6 Effects of exercise on articular cartilage & subchondral bone

Studies in young animals demonstrate that exercise is important in the adaptation, maintenance and integrity of osteochondral tissues (Arokoski et al., 2000; Brama, Tekoppele, Bank, Barneveld, & van Weeren, 2000; Deschner, Hofman, Nicholas, & Sudha, 2003; Otterness et al., 1998). Exercise shapes the biological properties and mechanical behaviour of AC and SCB which respond to mechanical load in a site-specific way (Appleyard et al., 2003; Arokoski et al., 2000; Brama, Bank, Tekoppele, & Weeren, 2001; de Grauw et al., 2006; Murray, Smith, Henson, & Goodship, 2001; Murray, Vedi, Birch, Lakhani, & Goodship, 2001; Young et al., 2005). Joint movement and mechanical load are necessary for postnatal development, the prevention of joint disease, and rehabilitation following injury (Little & Ghosh, 1997; R. J. Todhunter, 1996).

i) Articular cartilage

At birth, the composition of AC is homogenous (Brama, Tekoppele, Bank, Barneveld, & van Weeren, 2000). With development and rapid growth there are significant biochemical and biomechanical changes. Genetic factors, environment, exercise type and intensity influence the final quality of the musculoskeletal system (Arokoski et al., 2000; Little, Gosh, & Rose, 1997; Mienaltowski, Huang, Stromberg, & MacLeod, 2008; van Weeren, Brama, & Barneveld, 2000).

In young Beagles, adaptive changes in AC have been observed following moderate and long term exercise (Lammi et al., 1993). Vigorous physical activity during prepubertal and pubertal years in children has been associated with cartilage acquisition in the knee (Jones et al., 2003). In horses, functional adaptation of AC at different sites within the joint have been observed in response to weight bearing and exercise during the first 6 months of postnatal

life (Brama, Tekoppele, Bank, Barneveld, & van Weeren, 2000; van Weeren et al., 2000). These studies suggest that the adaptive response of AC to mechanical load may take place prior to attainment of skeletal maturity.

Daily exercise protects AC from degeneration and maintains homeostatic balance within the tissue (Otterness et al., 1998). This is in contrast to the negative effects of withholding exercise and mechanical load during early development (Otterness et al., 1998; van Weeren et al., 2000). A sedentary lifestyle in hamsters resulted in decreased PG content in AC and SF, cartilage fibrillation, pitting and fissuring (Otterness et al., 1998).

In dogs, joint immobilization caused a rapid reduction in AC thickness, PG synthesis and content (Brandt, 2003) and long-term immobilization caused long-lasting matrix changes in immobilized and contralateral joints despite the introduction of moderate exercise (Jortikka et al., 1997). However, these changes may be reversed if not prolonged (Haapala et al., 2001). Studies in the rat knee demonstrate that these changes may be associated with decreased mechanical stress and joint fluid circulation (Hagiwara et al., 2007).

Mechanical load influences chondrocyte biosynthesis and gene expression (Jones et al., 2003) and shapes the structural and functional properties of AC (Arokoski et al., 2000). *In vitro* studies demonstrate this is dependent on magnitude and duration of load. An increase in collagen type II and aggrecan mRNA has been observed in human chondrocytes subjected to increasing intermittent hydrostatic pressure (Ikenoue et al., 2003). Moderate (Parkkinen, Lammi, Helminen, & Tammi, 1992) and intermittent cyclic compression (Burton-Wurster, Vernier-Singer, Farquhar, & Lust, 1993) caused an increase in sulphate incorporation and PG synthesis in bovine and canine chondrocytes.

Arokoski demonstrated that in adult AC and SCB loading with physiological mechanical forces was necessary to maintain tissue homeostasis (Arokoski et al., 2000). Regular cyclic loading of AC increased PG synthesis and cartilage stiffness while continuous compression suppressed anabolic metabolism and

lead to tissue necrosis (Arokoski et al., 2000). Normal, moderate joint loading maintains joint health and function (Wong & Carter, 2003).

Microdamage to the collagen network and degenerative changes in AC and SCB are evident following strenuous exercise and cumulative stress (Appleyard et al., 2003; Brama, Tekoppele, Bank, Barneveld, Firth, et al., 2000; McIlwraith, 1996). Horses subjected to high intensity exercise regimens had cartilage lesions, fibrillation, PG loss and reduced tissue stiffness compared to the cartilage of horses subjected to low intensity exercise (Murray et al., 1999). High intensity training may lead to deterioration of AC and injury to SCB (Murray, Smith, et al., 2001; Murray et al., 1999).

Mechanical stimulus is an important contributor to the repair and regeneration of damaged cartilage. An increase in chondrocyte metabolism, collagen type II and PG synthesis was observed in experimentally induced OA in the canine model (Matyas, Huang, Chung, & Adams, 2002). *In vitro* synthesis of collagen type II was observed in mature rabbit AC and associated with tissue healing (Cheung, 1978).

The hypertrophic response of femoral head AC in dogs subjected to strenuous treadmill exercise was associated with an early repair response to injury (Vasan, 1983). However, the collagen component of mature AC has little capacity for remodeling due to its extremely low turnover rate (Stockwell, 1979b). In addition, early repair tissue resembling AC deteriorates progressively with time (Hunziker, 1999).

Deschner et al. (2003) demonstrated that the mechanical signals of exercise can promote degradation or repair of cartilage, dependent on the intensity of strain (Deschner et al., 2003). During exercise, signals of high mechanical strain stimulate cartilage destruction, whilst signals of low mechanical strain stimulate cartilage adaptation, synthesis and repair (Agarwal et al., 2004; Deschner et al., 2003). The effect of these signals can be observed in young growing horses where the exercise regimes to which they are subjected has lifelong repercussions for joint health and resistance to injury (van der Harst, 2005).

Determining the physiological capacity of joint adaptation to mechanical load during skeletal development, may assist in the design of training and exercise regimes which prevent DJD and promote tissue healing following injury. However, the response of cartilage or tendon to exercise *in vivo* is difficult to measure (Firth, 2006) and the intensity of strain required to bring about adaptive response in these tissues is unknown (Firth, 2006; McIlwraith, 2005).

ii) Subchondral bone

Exercise has a significant influence on skeletal development (P.A.J Brama, J.M Tekoppele, R.A Bank, A Barneveld, & P.R van Weeren, 2002; P.A.J. Brama et al., 2002; C. H. Turner, 1998). The adaptive response of bone to mechanical load can be observed by an increase or decrease in mass and geometry (Firth, 2004). Bone formation or resorption at a given site is determined by mechanical strain in terms of magnitude, rate, duration and direction (Lotz, Hashimoto, & Kühn, 1999; Woo et al., 1981) and is influenced by age, peak bone strain, gait and cyclic frequency (Nunamaker, Butterweck, & Provost, 1990).

Moderate long-term exercise causes an increase in bone quantity but not quality. For example, prolonged moderate exercise (65-80% maximum heart rate) in immature swine caused an increase in femur cortical thickness and volume without change in bone mechanical properties or composition (Woo et al., 1981). This adaptive response of bone increased load carrying capacity and energy absorption before failure. However, high intensity training in young growing animals inhibits growth (Woo et al., 1981).

Studies in human athletes reveal strain magnitude may be more important than the number of cyclic loads in controlling bone adaptation to load (Bennell et al., 1997). Osteogenesis is optimized when high-magnitude strain is applied at a high rate with few strain cycles. Activities such as weight training, sprinting and jumping have greater effect on bone mass and BMD than does endurance running. For example, Thoroughbred horses train at a pace much slower than racing speed and over time this may lead to low strain fatigue and loss of stiffness. With infrequent high speed training which loads bone differently, high

strain cyclic fatigue results in an increased incidence of fracture in 2 year old horses (Nunamaker et al., 1990).

Horses subjected to high intensity exercise had an increase in bone formation and SCB thickness and structural stiffness at loaded sites compared to low intensity exercised or unexercised horses (Firth et al., 2005; Murray, Vedi, et al., 2001). With high intensity exercise, osteoblasts lining the spongiosa recruit and up-regulate their activity and thickening of trabecular at the expense of the intra-trabecular spaces which become completely replaced with bone (Murray, Vedi, et al., 2001; Secombe et al., 2002). However, high intensity bone strain can cause bone matrix microdamage and initiate bone remodeling (Kawcak et al., 2001; R. B. Martin, 2000). Two year old thoroughbred horses subjected to high-speed treadmill exercise had an increase in SCB density which was also associated with lameness (Kawcak, McIlwraith, Nordin, Park, & Steyn, 2000).

Vascular penetration of the calcified cartilage through to hyaline AC has been observed in the distal third metacarpal of trained and untrained 2 year old thoroughbred horses (A. Boyde & Firth, 2004). The vascular canals may provide a pathway between bone and AC for extracellular fluid and the matrix degrading enzymes produced by osteoclasts. Repair of micro-cracks in the calcified cartilage layer with vascular invasion from the SCB may play a role in OA (Arokoski et al., 2000; Kawcak et al., 2001). Remodeling processes in SCB and calcified cartilage can cause biological and mechanical alteration to AC (Burr & Schaffler, 1997).

The response of AC and SCB to exercise, involves several pathways at the molecular level dependent on the intensity of strain. The expression of numerous genes involved in inflammation, tissue adaptation and remodeling (Figure 1.12). An understanding of the balance between joint adaptation and injury during high intensity training is necessary to increase understanding of exercise related DJD (Murray, Vedi, et al., 2001). The intimate relationship between AC and SCB and the influence of exercise during development has not yet been fully examined at the molecular level.

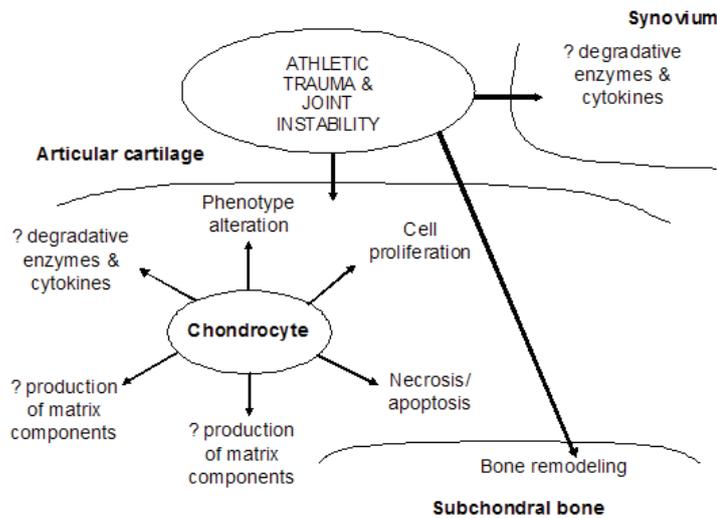


Figure 1.12 The mechanical signals of exercise

The mechanical signals of exercise affect all the osteochondral tissue and are mediated at the molecular level. In articular cartilage these signals induce a phenotypic change in articular cartilage chondrocytes, cellular proliferation, apoptosis, and the production of matrix catabolic and metabolic components including degradative enzymes and inflammatory cytokines. An adaptive response in bone leads to remodeling in subchondral mineralized tissues.

1.2.7 Gene expression

Trauma & inflammation

Recent cell culture studies suggest that the conversion of mechanical signals of exercise may be mediated through a common pathway with the activation of either cartilage destruction or repair (Deschner et al., 2003). The mechanical signals of exercise can promote degradation or repair of cartilage, dependent on the intensity of strain (Deschner et al., 2003). These signals are likely mediated by inflammatory cytokines; interleukin-1beta (IL-1 β) and tumour necrosis factor-alpha (TNF- α) and may be involved in the synthesis or degradation of collagen acting via a common pathway (Agarwal et al., 2004; Deschner et al., 2003; Perkins, Rogers, Firth, & Anderson, 2004). During exercise, signals of high mechanical strain stimulate cartilage destruction,

whilst signals of low mechanical strain stimulate cartilage adaptation, synthesis and repair (Agarwal et al., 2004; Deschner et al., 2003).

i) Cytokines

Cytokines are low molecular weight proteins involved in intercellular communication in multicellular organisms (Platt, 1996). These vital peptides are regulatory factors (PRF) with short to intermediate half-lives and high affinity for specific cell surface receptors. PRFs include the interleukins, TNF- α , growth factors and BMPs (Table1.3). PRFs have a range of biological effects acting through autocrine, paracrine or exocrine mechanisms and induce changes in gene expression.

Proinflammatory cytokines TNF- α and IL-1 β are produced by cells of the synovium, chondrocytes and mononuclear cells. These cytokines are central mediators in catabolic metabolism in AC and bone, and in the initiation of MMP gene expression (Hedbom & Hauselmann, 2002). TNF- α and IL-1 β can decrease chondrocyte synthetic pathways and upregulate degradative processes (McIlwraith, 1996).

Table1.3 The role of peptide regulatory factors involved in cartilage metabolism.

Sourced: Goldring (200a, 200b), Platt (1996).

Catabolic factors: Interleukin-1 α Interleukin-1 β Interleukin-17 Interleukin-18 Leukemia inhibitory factor Oncostatin M	Anabolic factors: Insulin-like growth factor-1 Insulin-like growth factor-2 Transforming growth factor- β (β 1, β 2, β 3) Fibroblast growth factors (2, 4, 8) Growth differentiating factor-5 Bone morphogenetic proteins (2, 4, 6, 7, 9, 13)
Inhibitors: Interleukin-4 Interleukin-10 Interleukin-13 Interleukin-1 receptor antagonist Interferon- γ	Regulators: IL-6 IL-8

TNF- α and IL-1 β are central mediators during inflammation, immunological responses and tissue injury (Bird et al., 2002; Moreland, 2003; Pelletier et al., 2001). TNF- α and IL-1 β can act alone or synergistically and both cytokines contribute to cartilage destruction (M. B. Goldring, 2000a). During OA the expression of IL-1, TNF- α and their receptors are increased in chondrocytes in association with MMP production (Moreland, 2003) and are targets for therapeutic intervention in OA (M. B. Goldring, 2000a).

Cytokine expression is an integrated and complex response dependent on tissue concentrations of TNF- α (Ostrowski, Rohde, Asp, Schjerling, & Pedersen, 1999). During strenuous exercise, an acute phase inflammatory response can be observed at the site of inflammation. This initiates a cytokine cascade and an influx of lymphocytes, neutrophils, macrophages and monocytes which support antigen removal and assist in tissue healing. The acute phase systemic response includes the expression of proinflammatory cytokines TNF- α , IL-1 β , IL-6, IL-10, as well as endogenous inhibitors (Ostrowski et al., 1999). Endogenous inhibitors include IL-1 receptor antagonist (IL-1ra), soluble type receptors of IL-1 (sIL-1r(I) and sIL-1r(II)) and the two soluble receptors for TNF- α (sTNF-r1 and sTNF-r2).

Inflammatory factors induced by IL-1 and TNF- α alone or in combination include: inducible nitric oxide synthase, cyclooxygenase 2 and phospholipase A2 (Goggs, Carter, Schulze-Tanzil, Shakibaei, & Mobasheri, 2003). These factors modulate chondrocyte function in OA and the spontaneous release of nitric oxide and prostaglandin E2 (M. B. Goldring, 2000b). Endogenous nitric oxide can suppress cartilage matrix synthesis and induce chondrocyte apoptosis (Lotz et al., 1999).

Other therapeutic targets of IL-1 and TNF- α may be the regulation of signal transduction pathway protein kinases regulated by chondrocytes (M. B. Goldring, 2000b). This includes stress-activated protein kinases: c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK), inhibitory kappa beta-1 (I κ β -1) and -2 kinases that release nuclear factor kappa beta (NF κ β) (Liacini, Sylvester, Li, & Zafarullah, 2002).

a) TNF- α

The catabolic role of TNF- α in cartilage matrix, may be in triggering proinflammatory events (Sandell & Aigner, 2001). Its influence in the pathological degradation of AC may be in the upregulation of IL-1 and IL-6 and inhibition of PG synthesis especially in the young (Platt, 1996). A decrease in collagen type I gene expression and increase in collagenase expression have been observed (Sandell & Aigner, 2001). TNF- α and IL-1 β produced a dose dependent decrease in mRNA levels of collagen type II (McIlwraith, 2005). IL-1 and TNF- α are believed to be key intermediaries in the pathogenesis of OA (Martel-Pelletier, 2004).

b) IL-1

Interleukin-1 β is involved in the maturation and activation of macrophages as well as cellular differentiation in bone and cartilage, and has been implicated in the degradation of AC (Liacini et al., 2002). IL-1 β is spontaneously released from OA cartilage but not normal cartilage (M. B. Goldring, 2000a; Moreland, 2003). The expression of IL-1 β can alter the homeostatic balance of extracellular matrix synthesis and result in rapid reduction of PGs and collagen in AC (Freen, Gettinby, & Lees, 2000). The metabolic response of AC to IL-1 β has been correlated with age. A shift from regulation of catabolic pathways in immature animals toward regulation of synthetic pathways in mature individuals has been observed in human and equine AC (Platt, 1996). This is likely related to a reduction in chondrocyte metabolic rate with skeletal maturity.

Interleukin-1 β can increase the synthesis of products involved in the degradation of connective tissues: proteinases (collagenase, gelatinase, proteoglycanase and plasminogen activator), prostaglandins and nitric oxide (M. B. Goldring, 2000b; Hedbom & Hauselmann, 2002). IL-1 β has stimulatory effects on collagen types I and III and suppressive effects of synthesis of cartilage specific collagen types II and IX *in vitro* (M. B. Goldring, Birkhead, Sandell, Kimura, & Krane, 1988; M. B. Goldring & Krane, 1987). This is characteristic of mineralized bone, inability of the cartilage matrix to repair following injury, and altered phenotypic expression of chondrocytes.

Interleukin-1 β secreted from macrophages enhances the production of several proMMP forms (zymogens) including: proMMP1 (interstitial collagenase), proMMP3 (stromelysin-1), MMP7 (matrilysin) and MMP9 (gelatinase B) (Sandell & Aigner, 2001). Once activated, the previously inactive precursors regulate the activity of IL-1 β but not that of IL-1 α (Ito et al., 1996). Prostaglandin E2, nitric oxide, interleukin-1 β and interleukin-6 respectively have been observed in cartilage explants and synovial fluids of rheumatoid arthritis (RA) and OA (Pelletier et al., 2001; Smith, 1999).

ii) Collagens

Collagens are ubiquitous proteins present in the extracellular matrix of connective tissues. Collagens give tissues structure and elasticity by providing a biomechanical scaffold for cell attachment and anchorage of macromolecules (Kadler, Holmes, Trotter, & Chapman, 1996). More than 27 collagen types exist in mammalian tissues. Gene regulation, cytokine induction and cell-matrix signaling govern the assembly of collagen fibres during embryonic growth, development, tissue healing and pathology (Williams, Gelman, Poppke, & Piez, 1978)

All collagen is synthesized within the cell and consists of three polypeptide chains (α -chains) which are formed on ribosomes along the rough endoplasmic reticulum (RER) (Kadler et al., 1996) (Figure 1.13). These collagen precursors (procollagen) are directed into the vesicular space of the RER and undergo hydroxylation and glycosylation of proline and lysine residues and the formation of the triple helical structure procollagen. Procollagens are converted into tropocollagens. Multiple tropocollagen molecules form fibrils. Multiple fibrils form collagen fibres. The triple helical fibril, approximately 300 nm in length and 1.5 nm in diameter, forms a rod flanked by amino (N) and carboxy (C) terminal propeptides regions. These regions are cleaved during extracellular processing by procollagen metalloproteinases. The short N and C telopeptides consist of 15-25 amino acids and are important to the tensile strength of the collagen fibres. Collagen fibers are stabilized by the formation of inter- and intra- molecular crosslinks by the actions of lysyl oxidase, an extracellular copper enzyme, which recognizes specific binding sites found in

type I, II and III collagen telopeptides and triple helix. The formation of immature and mature crosslinks is a continual process and can vary with tissue type. In AC extensive cross-linking is associated with material strength. In bone a significant pool of immature crosslinks exists and this is determined by bone tissue turnover rate.

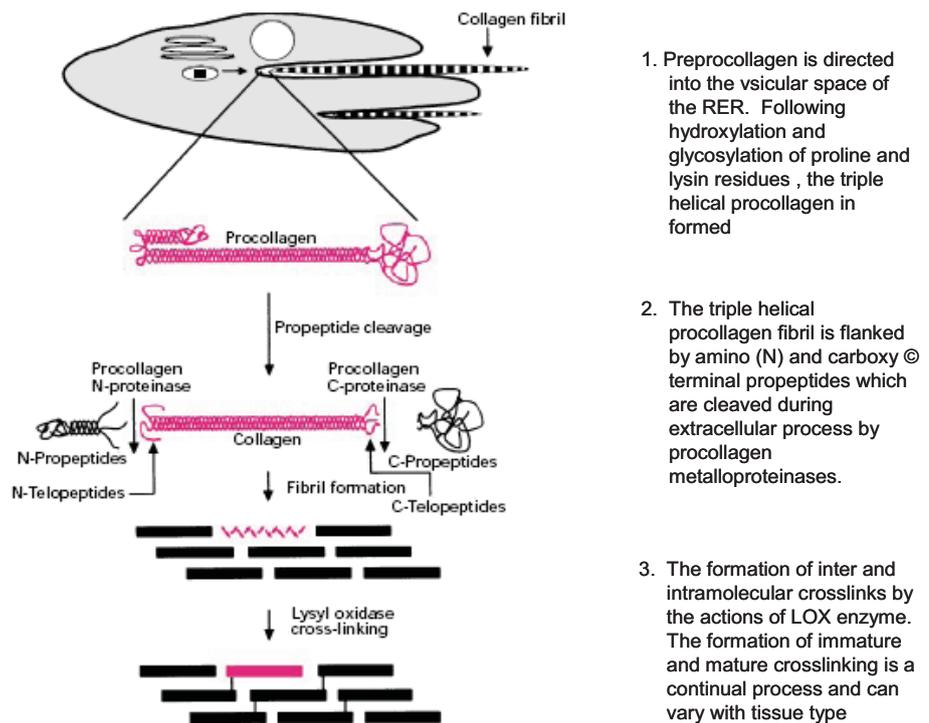


Figure 1.13 Collagen fibril formation

All collagen fibrils are formed within the cell as a result of entropic processes whereby soluble procollagens are converted into collagen by specific enzyme cleavage of propeptides by procollagen metalloproteinases. *Sourced: Kadler (1996) adapted.*

The fibril forming collagens (I, II, III, V and XI) are found in tissues able to resist tensile, shear and compressive forces including cartilage, bone, tendon, skin and blood vessels (Viguet-Carrin et al., 2006). They are characterised by a central triple helical region of α chains consisting of more than 300 glycine-X-Y repeats; where X and Y are derivative amino acids proline and hydroxyproline

and are modified post-translationally by enzymes requiring vitamin C as a cofactor during hydroxylation (Williams et al., 1978). Depending on the collagen type involved, α -chains can be homotrimeric or heterotrimeric (Viguet-Carrin et al., 2006).

Collagen fibrils are characterized by an axially staggered arrangement giving a characteristic light and dark band pattern when negatively stained and viewed by electron microscopy (Kadler et al., 1996). Fibrils may be arranged in complex three dimensional structures: orthogonal lattices, parallel bundles and concentric weaves (Viguet-Carrin et al., 2006; Williams et al., 1978).

a) Collagen Type I

Collagen type I consists of two identical α_1 chains and one α_2 chain [$\alpha_1(I)_2\alpha_2(I)$]. Collagen type I is found throughout the body excluding cartilage and is also synthesized in response to tissue injury (Kadler et al., 1996; Stokes et al., 2000). Bone is the richest source of collagen type I comprising 95% of its collagen content. Collagen type I enables bone mechanical properties and its ability to absorb energy generated during locomotion (Viguet-Carrin et al., 2006).

Contrary to earlier reports, collagen type I has been observed in the most superficial layer of normal AC and an increase in collagen type I expression has been observed in early OA and in deep calcified cartilage (Young et al., 2005). The expression of collagen type I in AC is an indicator of altered chondrocyte phenotype in human OA cartilage (Miosge, Hartmann, Maelicke, & Herken, 2004). The differences between studies may be related to variability among different individuals and also to limitation in the methods used. Northern blotting and *in situ* hybridization methods lack the sensitivity to detect or quantify low-level gene expression from tiny tissue samples. For this reason, amplification using semi-quantitative image analysis or real-time PCR may be used (Miosge et al., 2004).

b) Collagen Type II

The tensile properties of AC are attributed to the collagen type II triple helix. Denaturation of collagen type II in the superficial layer has been observed with

ageing and OA, extending to the deeper layers progressively with disease (Hollander et al., 1995). During early OA, an anabolic response of chondrocytes has been observed in the ovine meniscectomized model with increased expression of collagen type I and II (Appleyard et al., 2003; Young et al., 2005).

The regulation of mRNA levels in dense connective tissue is tissue-specific and influenced by skeletal maturity. An increase in mRNA expression for collagen type II has been observed in skeletally immature rabbit menisci with a decrease in mRNA expression of collagen type II with age (Le Graverand, Reno, & Hart, 1999). Similar observation has been observed in the neonatal growth cartilage of foals and is a reflection of the greater matrix synthesis in the young animal compared to adult AC (Johannessen, Skretting, Ytrehus, & Roed, 2007).

iii) Matrix metalloproteinases

The matrix metalloproteinases (MMPs, 23) are members of a super family of zinc dependent endopeptidases also designated matrixins. MMPs are critical factors to maintain tissue allostasis, regulate the extracellular environment and a diverse range of physiological and pathological processes. MMPs are active at neutral pH and hydrolyze components of the ECM including interstitial and basement membrane collagens, PGs and proteins. They are key mediators in embryogenesis, normal tissue growth and remodeling, wound healing and disease including arthritis, tissue ulceration, cancer, and cardiovascular and central nervous system pathology (Malemud, 2006a, 2006b). MMPs are required for normal bone development (Ortega, Behonick, Stickens, & Werb, 2003).

The MMP family includes disintegrin metalloproteinases (ADAMS), and disintegrin metalloproteinases with thrombospondin repeats (ADAMS TSs) (Figure 1.14). ADAMs are membrane bound MMPs and can degrade aggrecan, especially ADAMS-4 and ADAMS-5, during rheumatoid arthritis and OA (Malemud, 2006a). ADAM-17 is a TNF- α converting enzyme that activates pro-TNF- α (Malemud, 2006a).

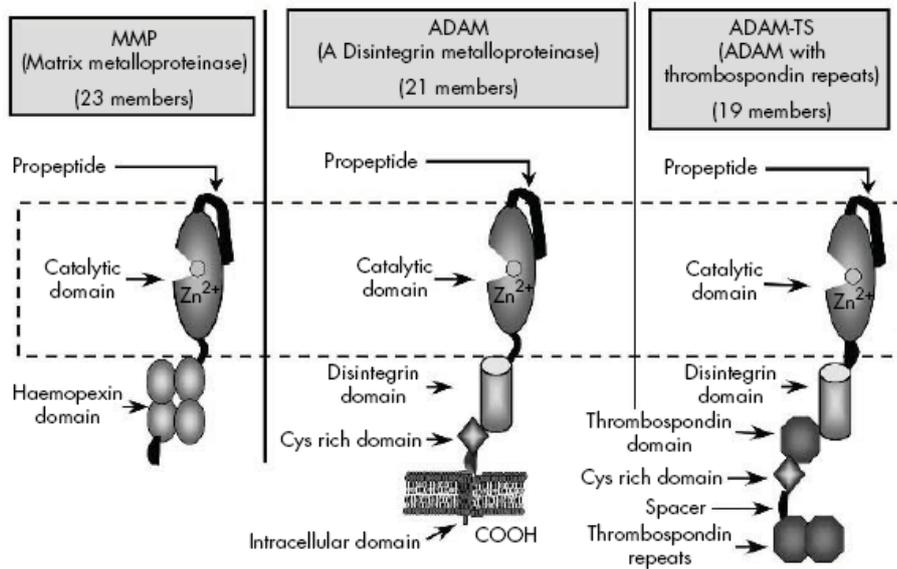


Figure 1.14 The zinc dependent endopeptidase families

Zinc dependent endopeptidase families include: Matrixmetalloproteinases (MMPs), disintegrin metalloproteinases (ADAMS), and disintegrin metalloproteinases with thrombospondin repeats (ADAMS TSs). The catalytic domain of these enzymes is dependent on zinc ion at the active centre. MMPs are secreted in zymogen form and are inactive in the presence of an N-terminal propeptide. Proteolytic removal of the propeptide, at cellular sites specific to individual proteinases, results in activation. MMPs have specific domains which confer specificity for substrate cleavage or cellular or extracellular matrix localization. Some members of the MMP family and all of the ADAMS family are membrane associated (MT-MMPs). MMP activity is inhibited by tissue inhibitor of metalloproteinases (TIMPs). MMPs cleave one or more components of the extracellular matrix and play a role in the pathology of cartilage and bone. Sourced: *Murphy & Lee (2005) adapted.*

The MMPs are divided into four sub-groups based on the substrate specificity: collagenases, gelatinases, stromelysins, and membrane type (MT) MMPs (Hooper, 1994; Johnson et al., 1998). Collectively these enzymes are capable of degrading the macromolecules of connective tissues. MMPs are synthesised in a latent form and activation is regulated by growth factors, endocrine and inflammatory cytokines, cellular transformation and tissue inhibitors of MMPs (TIMPs) (M. B. Goldring, 2000a; Malemud, 2006a). The synthesis of collagenase and/or stromelysin is often coordinated (Woessner, 1991).

Intracellular pathways involving stress-activated protein kinases and tyrosine-receptor protein kinases regulate MMP expression (Malemud, 2006a). In normal steady state tissue a low level of MMP activity is observed (Ito et al., 1996). An upregulated/activated state can be observed during physiological remodeling or inflammation (Johnson et al., 1998; Redman et al., 2005). A loss of activity control can result in arthritis, cancer, atherosclerosis, aneurysms, nephritis, tissue ulcers and fibrosis (Visse & Nagase, 2003).

MMPs are key regulators of inflammatory and anti-inflammatory processes. The axes of MMPs and cytokines may be intrinsically linked (G. Murphy & Lee, 2005). MMPs are upregulated in arthritis and are associated with elevated levels of IL-1 β and TNF- α and decreased levels of TIMPs (Malemud, 2006a). The involvement of MMPs in cartilage and bone pathology and irreversible joint destruction has led to the development of broad spectrum MMP inhibitors as potential therapeutic agents, with limited success (G. Murphy & Lee, 2005).

Within AC, the longevity of the ECM is dependent on a balance between anabolic and catabolic processes between tissue inhibitors of MMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) and MMPs (Smith, 1999). Specific stimuli such as cytokines, growth factors and ECM interactions (mechanical stress) are key regulators in these processes (G. Murphy & Lee, 2005).

An increase in MMP gene expression has been observed in OA. This includes an increase in MMP1, MMP2, MMP7, MMP8, MMP9, MMP13, MMP16 and MMP22, ADAMTS2, ADAMTS12, ADAMTS14, ADAMTS16, and TIMP3 (Sandell & Aigner, 2001; Smith, 1999). In particular, MMP1, MMP8 and MMP13 have a high affinity for collagen type II (Billinghurst et al., 1997). During RA, high levels of MMP1, MMP2, MMP3 and MMP9, MMP13 are observed in synovial tissues and fluids (Ito et al., 1996; Smith, 1999). Decreases in gene expression have also been reported and include MMP3, MMP10, ADAMTS1, ADAMTS5, ADAMTS9, ADAMTS15, TIMP1, and TIMP4. The Proteinases, MMP2, MMP3, have been implicated in the cleavage of large PG aggrecan (A. R. Poole, 1997). Increased levels of MMP2 and MMP9 have been observed in bovine SF during septic joint disease (Arican et al., 2000).

MMP2 and 3 are abundantly expressed in SF and cartilage in horses with naturally occurring joint disease (Trumble, Trotter, Oxford, & McIlwraith, 2001).

Recent studies suggest that MMPs can suppress inflammation by degrading biologically active cytokines such as IL-1 β . MMP1, MMP2, MMP3, and MMP9 secreted from stimulated connective tissue cells, degrades IL-1 β but not IL-1 α (Ito et al., 1996). Thus, MMPs are both effectors and regulators of inflammation (McQuibban et al., 2000).

a) Gelatinases

The gelatinases MMP2 and MMP9 are unique enzymes which actively degrade denatured collagens and gelatins as well as aggrecan. ProMMP2 can be localized in many tissues and is catalytically activated by MT1-MMP (Ortega et al., 2003). Active MMP2 digests collagen types I, II and III (Visse & Nagase, 2003). MMP2 and MMP9, and MT1-MMP, are involved in angiogenesis (Ortega et al., 2003). MMP2 is important for osteogenesis (Visse & Nagase, 2003). Although MMP2 null mice develop without clinical abnormality, MMP2 mutations in humans results in an autosomal recessive form of multicentric osteolysis. This rare genetic disorder causes destruction and resorption of the affected bones (Visse & Nagase, 2003). MMP2 plays a role in osteoclastic resorption in pathological conditions (G. Murphy & Lee, 2005).

1.2.8 Methods in assessing articular cartilage & subchondral bone

Understanding the mechanisms of bone homeostasis and DJD has been dependent on the use of *in vivo*, *ex vivo* and *in vitro* experimental settings. Methodological approaches allow insight into the mechanisms driving bone and cartilage degradation at the cellular, organ and organism level. Advantages and limitations exist within each experimental setting. The development of a wide array of biochemical assays over the last decade has given further insight in to the mechanisms of skeletal homeostasis. The development of cell culture, cartilage explants and animal models has become a rapidly growing area of research.

i) In Vivo

a) Live imaging

In vivo studies rely on non-invasive technologies to examine pathology or potential threat of DJD. Current technologies to noninvasively measure bone material properties *in vivo* in large animals are limited (Table 1.6). Clinical observation and physical examination are initially used to identify pain, gait or joint abnormality. Radiographic imaging, magnetic resonance imaging (MRI), ultrasonography (US), computed tomography (CT), or dual energy x-ray absorptiometry (DXA) can determine bone mineral content and density. However, assessment of AC integrity is difficult (H. Brommer, Rijkenhuizen, Brama, Barneveld, & Weeren, 2004).

Radiographic imaging is the gold standard for diagnosing OA (joint space width) following changes in SCB (Garnero, Rousseau, & Delmas, 2000) including changes in density, trabecular detail and cortical thickness. However, bone loss must exceed 30% before becoming evident radiographically (Kawcak et al., 2000; Vanderperren et al., 2009). Changes to synovial membrane and AC are not visible radiographically making it unsuitable as a preventative diagnostic tool due lack of resolution and large precision error (Kawcak et al., 2000).

Table 1.4 Technologies to measure bone mineral density (BMD) and bone strength in large animals.

Sourced: Lepage (2001); McLuree et. al., (2001); Vanderpeeren et. al., (2009).

Technology to measure bone mineral density	Units
Dual-energy x-ray absorptiometry (DEXA)	BMC g/cm ²
Single energy x-ray absorptiometry (SXA)	BMC g/cm ²
Radiographic absorptiometry (RA)	RBAE mm Al
Quantitative computed tomography (qCT)	BMD, g/cm ³
Quantitative ultrasonography (QUS) (across axis)	SOS m/s
Multi-site QUS (along axis)	SOS m/s

Whilst arthroscopy is a widely used method to determine AC *in vivo*, correlation of arthroscopic scoring, radiographic change and real damage have been reported as poor in the equine carpal (Kannegieter, Burbridge, & Alwan, 1991), to good in the stifle (Steinheimer, McIlwraith, Park, & Steyn, 1995), and variable in the first phalanx (H. Brommer, Rijkenhuizen, et al., 2004). This may be because the visible area is not representative of the entire articular surface (H. Brommer, Brama, & Weeren, 2004).

b) Molecular markers

Biochemical markers have become increasingly used for the assessment of bone metabolic activity. Highly specific markers can detect early changes at the cellular level prior to the development of irreversible disease (Dias et al., 2008; Fox & Cook, 2001; Fuller et al., 2001; Garnero et al., 2002; Garnero et al., 2000; Lepage et al., 2001). Molecular markers for bone formation and osteoblastic activity in serum and urine, include isoforms of bone specific alkaline phosphatase (BALP), osteocalcin (OC) and procollagen type I C- and N-terminal propeptides (Dias et al., 2008; Fuller et al., 2001; Lepage et al., 2001). A positive correlation between BALP and AC degradation has been observed in equine SF during OA (Fuller et al., 2001).

ii) Ex vivo

Macroscopic observation at necropsy is routinely used to determine joint pathology. Macroscopic, histological and biomechanical assessment of AC and bone are common tools to investigate the pathogenesis of DJD (McIlwraith, 2005).

Quantitative assessment and classification of the articular surface can be evaluated with the cartilage degeneration index (CDI). This can be done with the application of Indian ink to the entire articular surface and computer controlled analysis of stained and non-stained cartilage surfaces referenced to a gray scale calibration (Harold Brommer, Weeren, & Brama, 2003).

With polarized light microscopy, a loss of birefringence intensity in AC is associated with a loss of collagen or change in orientation within the superficial zone (Appleyard, Ghosh, & Swain, 1999). Changes in collagen birefringence are an indication of OA and correlated with biomechanical properties including

dynamic shear modulus and macroscopic grade (Oakley et al., 2004). Electron microscopy (scanning and transmission) allows 3-dimensional imaging of collagen fibers and chondrocytes (Jeffery, Blunn, Archer, & Bentley, 1991; Kääh, Richards, Walther, ap Gwynn, & Nötzli, 1999).

Histological assessment of AC with Safrin-O/fast green is widely used to assess the efficacy of the collagen network and PG content. Histopathological grade of severity of cartilage damage is evaluated by the Mankin score (0-14 point systems) and correlated with the metabolic state of chondrocytes during OA development (Appleyard et al., 1999; Dorotka et al., 2005). Immunohistochemical PG staining with antibodies is also widely used to determine chondrocyte metabolism (Appleyard et al., 1999).

iii) *In vitro*

The mechanical properties of AC and bone can be assessed *in vitro* under differing loading conditions. Local stimulation of PG content and cartilage synthesis have been observed following dynamic compression in cartilage and bone (Burton-Wurster et al., 1993; Parkkinen et al., 1992). Repetitive loading of AC produces variable responses in collagen and aggrecan content dependent on loading technique, frequency, duration and the range of forces applied (Smith et al., 2000). A comparison of dynamic force transmitting properties of AC and SCB reveal that alteration in the quality of SCB could have a significant effect on the ability of SCB-AC system to withstand compressive force *in vivo* (Radin, Paul, & Lowy, 1970).

Cell culture studies allow the investigation of cellular process without the need for large quantities of biological sample (P. G. Murphy & Hart, 1993). As a result *in vitro* investigations have allowed the study of chondrocytes and ECM metabolism (McQuillan et al., 1986; Tyler, 1989). However, limitations of this technology include the lack of support from matrix interactions, phenotypic changes, and differential gene expression has been observed in many species (Achnabel et al., 2002; Bianco, Cancedda, Riminucci, & Cancedda, 1998) and may not reflect accurate changes *in vivo* (Bau et al., 2002).

1.2.9 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) based methods are commonly used to detect DNA and RNA (Bustin & Nolan, 2004). PCR is an uncomplicated technique that amplifies a DNA template to create specific DNA fragments *in vitro*. The convenience of this method is the ease with which amplification of genomic or transcriptional information can be done. Millions of copies of DNA can be amplified with a high degree of precision and reliability within hours. PCR is widely used in diagnostic laboratories, forensic research and has facilitated the advancement of cloning technology. PCR allows detection, amplification, extension and quantification in real-time using a very small amount of biological. PCR is sensitive enough to enable quantitative amplification of RNA from a single cell, material making PCR preferential to microarrays which require greater quantities of total RNA (Kubista et al., 2006). PCR involves a repetitive cycle consisting of three phases: denaturation, annealing and extension. A variation of methods can be used and include: reverse transcription (RT-PCR), real time (rt-PCR), semi-quantitative and quantitative.

The outcomes of RT-PCR are highly dependent on the reaction parameters (Meijerink et al., 2001). These include: extraction and isolation of high quality total RNA free from ethanol or genomic contamination; the use of carefully designed primers at appropriate concentrations with similar melting temperatures, and which have met specific design requirements. The use of magnesium, dNTPs and enzymes at optimal concentrations are critical (Bustin & Nolan, 2004). In addition, the selection of standardized internal controls against which gene expression can be normalized needs to be done with consideration of tissue type and developmental stage (Miosge et al., 2004). This is due to reported variability of housekeeping genes with different phases of cell growth, cycle and differentiation. The validity of data is based on the integrity and optimization of the components utilized in the analysis (Fleige & Pfaffl, 2006; Fontanesi, Colombo, Beretti, & Russo, 2008). Inappropriate primer design, non-sterile technique, or simply inadequate mixing of components is sufficient enough to produce negative or inaccurate data.

1.2.10 Animal models

Although OA is the most common DJD affecting humans, it has not been possible to characterize the sequence of events which lead to OA in human joints. This is due to difficulty identifying early changes in OA and access to joint tissue from patients in early stages of the disease. For this reason animal models have been widely used to investigate the pathogenesis of joint disease. Animal models allow researchers increased control over experimental processes and the ability to examine differences between young, aged, surgically induced and spontaneous joint disease in exercised and unexercised animals (Bendele, 2001). This allows the onset of pathology and disease progression to be investigated. In addition, the efficacy of therapeutic agents can be determined in the whole animal. However, unpredictability of disease onset, complexity of the animal model, species differences and increased expense compared with *in vitro* techniques are disadvantages (May, 1996).

Rodents are a widely used model in orthopedic disease due to low cost and large background data (A. S. Turner, 2002). However, histoanatomical and structural differences are observed in rat bone compared to other quadrupeds and primates (Bagi, Wilkie, Georgelos, Williams, & Bertolini, 1997; Jowsey, 1966). Dogs, rabbits, guinea pigs and primates have also been used (Anderson-MacKenzie et al., 2005; Cheung, 1978; Haapala et al., 2001; Huebner et al., 1998; Panula, Helminen, & Kiviranta, 1997; Pritzker, 1994).

Many orthopedic diseases affecting humans and other veterinary species spontaneously arise in sheep (Murray, 2002; A. S. Turner, 2002). Sheep have been used as an experimental model in orthopedic studies to assess bone formation, fracture healing and disease (Dias et al., 2008; Lill, Gerlach, Eckhardt, Goldhahn, & Schneider, 2002; A. S. Turner, 2002).

The ovine meniscectomy model has been widely used to investigate changes associated with initiation and progression of OA (Appleyard et al., 2003; Oakley et al., 2004). The availability of the ovine model, ease of handling, maintenance, cost and similar kinetics to human skeletal turnover, make the ovine model favorable for orthopedic research (A. S. Turner, 2001, 2002).

1.3 Summary

Osteoarthritis is the most common DJD affecting terrestrial vertebrates (Bhosale & Richards, 2008). It is characterized by slow and progressive cartilage loss, SCB remodeling, soft tissue inflammation, pain and mobility loss (Felson et al., 2000). Many mammals develop OA spontaneously and by 65 years of age more than 50% of adult humans are afflicted (M. B. Goldring, 2006). Despite the prevalence of the disease, the etiology of OA remains poorly understood (Martel-Pelletier, 2004).

Traumatically induced joint disease and lameness are major causes of poor performance and early retirement of the equine athlete (Jeffcott, Rosedale, et al., 1982; Pool, 1996; Schlueter & Orth, 2004; R. J. Todhunter, 1996). Injury, normal and abnormal stressors, genetics and age can initiate changes within the synovial joint (McIlwraith, 1996). In horses, these changes can include inflammation of the synovium, degradation of AC and sclerosis of the underlying SCB (Cantley et al., 1999). The tarsus is the most commonly affected hindlimb region associated with lameness in the horse (Vanderperren et al., 2009).

The resilience of AC to withstand mechanical strain is dependent on the structural organization of ECM macromolecules and chondrocyte viability (Brama, Tekoppele, Bank, Barneveld, Firth, et al., 2000). Damage to the collagen network can cause chondrocyte injury, disrupt ECM metabolism and failure to maintain PG content. An increase in bone turnover and sclerosis of SCB are important features of DJD (Hayami et al., 2006).

The local actions of IL-1 β and TNF- α have been associated with cartilage degradation and the initiation of inflammatory processes. A catabolic response to injury outweighs synthetic processes and results in progressive degeneration of the ECM and loss of cartilage (M. B. Goldring, 2000b).

Matrix metalloproteinases play an important role in the turnover of cartilage ECM under normal and pathological conditions (Nguyen et al., 1993). During growth and skeletal development, the degradative process is balanced by the

expression of proteinases and their biological inhibitors (M. B. Goldring, 2000a). In normal adult tissue, MMP activity is very low and the ECM is in a state of dynamic equilibrium. This is in contrast to the upregulated/activated state observed during physiological remodeling or inflammation (Johnson et al., 1998).

Exercise is necessary for the maintenance of healthy AC (Otterness et al., 1998). The mechanical signals of exercise can promote adaptation, repair, or degradation of AC dependent on the intensity of strain (Deschner et al., 2003). Exercise of a moderate intensity and cyclic load rate cause an increase in PG synthesis and content, and increase in stiffness of the collagen fibrillar network (Roos & Dahlberg, 2005). Joint disuse and exercise intensity that exceeds physiological constraint result in cartilage fibrillation, pitting, fissuring and gradual deterioration of the matrix and collagen network. These signals are likely mediated by inflammatory cytokines acting via a common pathway (Deschner et al., 2003) and the balance between MMPs and their tissue inhibitors (Hooper, 1994; Hulejová et al., 2007).

This research will be the first to examine gene expression in AC and SCB *in vivo* in unexercised and exercised pasture maintained Perendale sheep. The outcomes of this study will contribute to the limited knowledge of the signaling events that regulate the adaptation and growth of AC and SCB under the influence of exercise and developmental age.

1.4 Hypotheses

1.4.1 Objectives of this study

The purpose of this study was to conduct a molecular investigation on the effects of exercise and developmental age on gene expression in AC and SCB bone in Perendale sheep. However, limited protocol existed for the total isolation of RNA from small samples of cartilage and bone in the ovine model. As a result, the focus of this research became the development of analytical protocols for gene expression analysis in ovine osteochondral tissues, and the application of these protocols in a pilot study to examine the effects of exercise and development on gene expression. This study used RNA isolated from a subset of Perendale sheep involved in an earlier study investigating the effects of exercise on the musculoskeletal system. RT-PCR was a cost efficient and effective technique for gene expression analysis to uphold or reject the hypotheses that:

- Gene expression in AC and SCB of the intermediate ridge of the distal tibia of the ovine tarsal joint, from exercised and unexercised sheep, will be significantly different
- In the unexercised control sheep there will be an increase in cytokine IL-1 β , TNF- α , matrix degrading enzyme MMP2, and collagen types I and II gene expression in AC and SCB
- Where gross joint morphology demonstrated moderate or severe cartilage erosion, there would be a consistent increase in inflammatory gene expression and matrix degrading enzyme MMP2.

Analysis and interpretation of semi-quantitative data was carried out in context of previous and future investigations to identify the impacts of exercise on joints in veterinary and human health. An outline of the methodology used for development of the RNA isolation protocol is given in chapter 2. Chapter 3 presents the findings of the exercise trial in regards to gene expression in AC and SCB from exercise trained and unexercised control sheep and provides discussion in context of the relevant literature. Chapter 4 provides an overall discussion of the research findings, outlines limitations of the present study and suggestions for future research.

Chapter 2

Optimisation of RNA extraction protocols from articular cartilage and subchondral bone for gene expression analysis in joint disease

Pedley, R.M., Howe, L., Rogers, C., Smith, A.

2.1 Abstract

Osteoarthritis is the most common DJD affecting terrestrial vertebrates (Bhosale & Richards, 2008) and is characterized by slow and progressive cartilage loss, SCB remodeling, soft tissue inflammation, pain and mobility loss (Felson et al., 2000). Despite the prevalence of OA the etiology of the disease remains poorly understood (Pelletier et al., 2001; Schlueter & Orth, 2004). Gene expression analysis in cartilage and bone through the isolation of RNA and reverse transcription polymerase chain reaction (RT-PCR) has increasingly become the diagnostic and prognostic tool of choice to further our understanding of joint disease (Bosma et al., 1998; Copois et al., 2007; I. Martin et al., 2001; Matyas et al., 2002; Rai et al., 2008; Raj, Moreno, & Gomella, 1998) However, the isolation of good quality RNA which is free from RT-PCR inhibitors is critical for this evaluation and presents a major technical challenge

for researchers (Fleige & Pfaffl, 2006; Ireland, 2003; McKenna et al., 2000; Reno, Marchuk, Sciore, Frank, & Hart, 1997; Vandesompele et al., 2002). In this pilot study, we report a simple, fast and reliable method for the rapid isolation of microgram per gram amounts of purified RNA from young and skeletally mature animals. This new RNA isolation protocol reliably and consistently isolates high quality RNA free of RT-PCR inhibitors from ovine SCB and AC, as well as cellular tissues, and allows the recovery of total RNA from very small quantities of tissue making it suitable for gene expression analysis when only a limited quantity of biological material is available.

2.2 Introduction

Osteoarthritis is a heterogeneous group of conditions that culminate in defective AC and bone loss. OA is characterized by slow and progressive cartilage deterioration, SCB remodeling, inflammation of the synovium, pain and increasing disability (Pelletier et al., 2001; Schlueter & Orth, 2004). Many mammals develop OA spontaneously (Huebner et al., 1998), for example, by 65 years of age more than 50% of people are afflicted with OA (M. B. Goldring, 2006). In addition, OA causes significant financial loss to animal production and sporting industries (Arican et al., 2000). In sport and racing horses, OA and lameness are the most important causes of poor performance, early retirement, or euthanasia of the equine athlete (Jeffcott, Rosedale, et al., 1982; R. A. Poole, 1996; Schlueter & Orth, 2004; R. J. Todhunter, 1996). Despite the prevalence of OA in the human and animal populations and the effect on health, welfare and economic cost (Arican et al., 2000; Hernandez & Hawkins, 2001; Jeffcott, Dalin, et al., 1982), the etiology of the disease remains poorly understood (Pelletier et al., 2001; Schlueter & Orth, 2004).

Examination of cartilage and bone metabolism *in vivo* through gene expression can further our understanding of joint disease (Matyas et al., 2002). However, the isolation of intact RNA from AC and bone, free from RT-PCR inhibitors, is a major technical challenge in gene expression analysis (Ireland, 2003; Reno et al., 1997). Bone is composed of an abundant mineralized matrix making it difficult to isolate RNA (Ireland, 2003). Dense hypocellular connective tissues

such as AC, ligament and tendon, are resistant to disruption due to the highly cross linked collagen and PG content of the extracellular matrix (Adams, Quiu Huang, Yue Uao, & Sandell, 1992). In addition, negatively charged PGs co-purify with the RNA extract and contaminate downstream applications (Chomczynski & Sacchi, 1987). As a result, low quantities of RNA are recovered from normal AC and lesser amounts are recovered from arthritic tissue (Clements et al., 2006).

Several methods have been described in avian and mammalian species, using fetal and mature tissue, to address some of these problems (Adams et al., 1992; Chomczynski & Sacchi, 1987; Leboy, Shapiro, Uschmann, Oshima, & Lin, 1988; MacLeod, Burton-Wurster, Gu, & Lust, 1996; Reno et al., 1997). Leboy *et. al.* (1988) successfully isolated RNA from mineralized avian bone for Northern blot and *in situ* hybridization, by double extraction using cesium chloride density centrifugation and guanidine HCl-ethanol precipitation and hot phenol extraction (Leboy et al., 1988). However, this method was too labor intensive to be practical for multiple sample work (Adams et al., 1992) and whilst *in situ* methodology is valuable for qualitative gene analysis during development, differentiation and growth (Sandberg & Vuorio, 1987), it is not quantitative. In addition, Northern blot requires samples to be pooled from a joint surface to obtain sufficient RNA (Matyas et al., 2002).

Adams *et. al.* (1992) examined different methods of tissue collection and homogenization. This included cryosection and scraping cartilage samples directly from canine stifle and shoulder joints. Whilst cryosection gave the greatest yield of RNA, the method was tedious, time consuming and increased the risk of RNA degradation (Adams et al., 1992). Different methods of mechanical disruption have been examined such as: manual chopping, pulverization by mortar and pestle or polytron (Adams et al., 1992), freezer milling (Marchuk, Sciore, Reno, Frank, & Hart, 1998; McKenna et al., 2000) and liquid nitrogen cooled Braun Mikro-Dismembrator Vessel (Reno et al., 1997; Young et al., 2005). However, variability exists between studies and the need for large quantities of starting material make these methods less suitable for small quantities of cartilage and bone samples.

Cell culture studies overcome the need to increase the quantity of starting tissue (P. G. Murphy & Hart, 1993). As a result, much of the published data on AC is based on RNA obtained from chondrocytes *in vitro*. However, differential gene expression and phenotypic changes have been observed in human (Achnabel et al., 2002; Bianco et al., 1998), as well as equine and canine articular chondrocytes (MacLeod et al., 1996) and may not reflect accurate changes *in vivo* (Bau et al., 2002). For example, high levels of proteinases, enzymes and IL-1 β expression are observed in normal and osteoarthritic AC *in vitro* but not *in vivo* (Bau et al., 2002).

The aim of this study was to evaluate the effectiveness of two methods of mechanical tissue disruption and two methods for RNA isolation, to provide high quality RNA from small quantities of AC and SCB samples. A new protocol adapted from several published protocols was used as well as a commercially available kit

2.3 Materials and methods

An outline of the methodology used for extraction of total RNA from ovine tissue suitable for gene expression analysis is shown in Appendix 1.1.

2.3.1 Tissue preparation

Skeletally mature (2 and 3 year old) and immature (16 week old and 1 year old) male and female sheep were stunned by captive bolt and euthanized by exsanguination in local abattoir or the post mortem facility at Massey University, Palmerston North in accordance with the regulations set out by the Massey University Animal Ethics Committee, Palmerston North, New Zealand. Within 15 minutes of euthanasia, soft tissue dissection was performed. Samples were collected from the right lobe of the liver (20-50mg), pregnant uterine horn (40mg), and whole joints (metacarpal and tarsal) of the left hind leg. Tissues were removed using aseptic technique and rinsed in sterile, RNase-free phosphate buffered saline (Sigma, St Lewis, MO, USA) to remove adherent blood and blotted on filter paper (Clements et al., 2006). The skin was removed from the limb tissues. All tissue samples were snap frozen in liquid nitrogen

and kept on ice for less than 60 minutes until transfer into a -80°C freezer to ensure RNA stability in dense connective tissues and cellular tissues post-mortem (Fontanesi et al., 2008; Marchuk et al., 1998). All tissues remained at -80° until RNA extraction was performed (Yasojima, McGeer, & McGeer, 2001).

2.3.2 Limb dissection

The frozen intact joints were removed from the -80°C freezer (Appendix 2.1) and maintained on ice for 45-60mins. The frozen samples were immobilized in a small bench-top vice. Gross lateral dissection through the tendons of mm. gastrocnemius et flexor digitorum superficial, m. flexor digitorum profundus, m. extensor digitorum lateralis and m. peronaeus tertius were performed with a number 11 scalpel blade. Severance of the origin of the peronaeus tertius, medial digital extensor and long digital extensor muscles allowed exposure of the synovial tarsal joint. Gross lateral dissection through the extensor and flexor muscle and tendons of the digitorum lateralis, profundus and suspensory ligament allowed exposure of the distal metacarpal and first phalanx.

During gross dissection, 10-100mg samples of extensor tendon, extensor muscle, collateral ligament and synovial membrane were taken. On opening the joint capsule, the tissue was snap frozen in liquid nitrogen to prevent mRNA degradation due to thawing (Yasojima et al., 2001). A general description of the joint surface and photograph were taken (Figure 2.1).

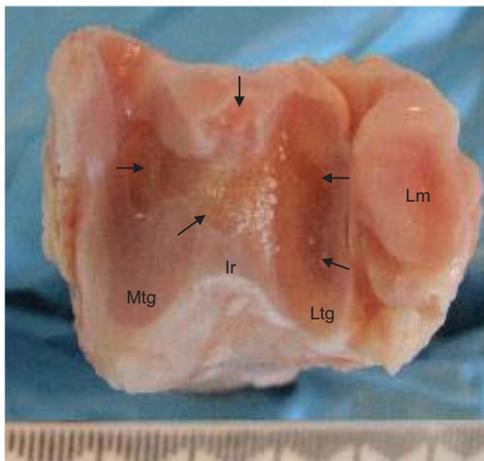


Figure 2.1 The distal tibia from a two year old ewe following gallop exercise.

The distal tibia from a two year old ewe which had been subjected to gallop exercise 8 months prior to euthanasia. There is wide spread cartilage erosion (arrows) extending from the medial trochlea groove (Mtg), across the intermediate ridge (Ir) and into the lateral trochlea groove (Ltg). No cartilage erosion is evident on the lateral malleolus (Lm).

Synovial fluid samples 50-100mg were collected from the intermedial grooves and from regions of cartilage erosion where synovial fluid had accumulated. Articular cartilage samples (5-50mg) were harvested from lateral and medial condyles of the proximal surface of the talus with a zesting tool (Appendix 3.1) and from the intermedial grooves with a sterile number 11 scalpel blade. AC samples were harvested from the corresponding regions of the distal tibia. SCB was harvested from the lateral and medial condyles and intermedial grooves of the distal tibia with sterile number 10 and number 11 scalpel blades, respectively. SCB samples were also harvested from the corresponding regions of the talus.

All AC and SCB samples were snap frozen in liquid nitrogen, weighed and placed into sterile 1.5ml eppendorf tubes. Eppendorf tubes were sealed, kept on ice and placed temporarily into a -20°C freezer (less than 1 hour) (Yasojima et al., 2001), then transported on ice to be stored at -80 °C until required for RNA extraction (Marchuk et al., 1998; Yasojima et al., 2001).

2.3.3 RNA Extraction

Two methods of mechanical disruption (mortar and pestle or stainless steel ball bearings enclosed with a sterile eppendorf tube) and two RNA extraction methods (a commercially available kit (RNeasy® Micro Kit™, Qiagen, Victoria, Australia) and an adaptation of a manual method of RNA isolation (Chomczynski & Sacchi, 1987; Heinrichs et al., 1997)) were compared to determine which method would isolate sufficient total RNA for gene analysis from small quantities of tissue sample.

a). Tissue disruption and homogenization

Two mechanical disruption methods were evaluated to determine which method would consistently yield the highest quality RNA from AC and bone samples (5-20mg). For the samples undergoing RNA extraction with the RNeasy® Micro Kit™, the frozen samples were pulverized in liquid nitrogen by either mortar and pestle, or with 3-5 sterile RNase-free stainless steel ball bearings (3-4mm) enclosed within a sterile eppendorf tube and vortexed vigorously for 4-7mins. For the samples undergoing RNA extraction with the manual method, the frozen tissue sample (10-60mg) was suspended in a solution (20ul/mg of tissue wet weight) containing 4M guanidine isothiocyanate (Sigma, St Lewis, MO, USA), 25mM sodium citrate (pH 7.0), and 0.1M 2-mercaptoethanol (Sigma, St Lewis, MO, USA). Tissues were disrupted by either mortar and pestle, or with 3-5 sterile RNase-free stainless steel ball bearings (3-4mm) enclosed within a sterile eppendorf tube and vortexed vigorously for 4-7mins until homogenized.

b). RNeasy® Micro kit™

Total RNA was prepared from AC, SCB and liver using the RNeasy® Micro Kit™ and on column DNase digestion (Qiagen, Victoria, Australia), according to the manufacturer's instructions. Briefly, following mechanical disruption, the homogenate was suspended in RLT buffer and passed through a QIA shredder (Qiagen, Victoria, Australia) and underwent proteinase K digestion (Qiagen, Victoria, Australia). The eluted lysate was mixed 0.5 volumes with 96-100% ethanol, applied to an RNeasy MiniElute Spin column (Qiagen, Victoria, Australia) and centrifugation (10000g at 4°C for 15s). After washing and DNase

digestion the RNA was eluted in 20ul of RNase-free water (Sigma, St Lewis, MO, USA).

c). Manual RNA isolation

Total RNA was prepared from all tissues using an adaptation of the Chomczynski and Sacchi method of RNA isolation (Chomczynski & Sacchi, 1987; Heinrichs et al., 1997)

i). Tissue digestion

Following tissue disruption, tissue homogenization, all disrupted homogenates were then diluted (15ul/mg of tissue wet weight) with a solution containing 19mM Tris-HCl (pH 7.0) and 0.72g/liter proteinase K (Sigma, St Lewis, MO, USA), and digested for 60mins at room temperature to allow the complete dissociation of nucleoprotein complexes.

ii). RNA extraction & phase separation

RNA extraction was carried out with phenol (USCB Corp., Cleveland, Ohio, USA) and chloroform (Sigma, St Lewis, MO, USA) (1:1) (using 100ul of phenol or chloroform per 10mg of tissue wet weight). Tubes were vortexed vigorously and incubated at room temperature for 15mins before centrifugation (12000g at 4°C for 15mins) to pellet the tissue residue. The aqueous phase (approximately 400-600ul) was recovered, diluted 1:2 with 3M sodium acetate (pH 5.2) (Sigma, St Lewis, MO, USA) (1:2) and mixed well by vortexing.

iii). RNA precipitation

RNA was precipitated on ice for 30mins in RNase free iso-propylalcohol (Sigma, St Lewis, MO, USA) (adding at least 1ml of RNase-free iso-propylalcohol per 0.75ml phenol used for the initial homogenization) to ensure removal of PCR inhibitors (Hanni, Brousseau, Laudet, & Stehelin, 1995). The precipitate was collected by centrifugation (12,000g for 30mins at 4°C) and the supernatant discarded.

iv). RNA wash

The pellet was washed with 75% RNase-free ethanol (Sigma, St Lewis, MO, USA) (adding at least 1ml of 75% RNase-free ethanol per 0.75ml phenol used for the initial homogenization). The sample was mixed by vortexing and collected by centrifugation (7500g for 5mins at 4°C). The supernatant was discarded and the pellet air dried at room temperature under a bench top lamp for 10-15mins. Care was taken to avoid complete drying of the pellet to ensure solubility.

v). RNA solubilization

The pellet was resuspended in 20ul of RNase free water (Sigma, St Lewis, MO, USA) and incubated at 55°C for 10mins.

2.3.4 RNA quality evaluation

RNA concentration was measured by absorbance at 260nm on a NanoDrop ND-1000 Spectrophotometer™ (NanoDrop Technologies Inc., Wilmington, DE, USA) and the concentration was adjusted to account for the dilution. Total RNA quality was visualised by gel electrophoresis using approximately 1ug of RNA on 1% agarose gel in 1 x TAE buffer running at 100V for 40mins. RNA was visualized (Appendix 4.1) with ethidium bromide stain and imaged under UV light by Gel Doc 2000 and Quantity One imaging and analyzing 1-D software (BioRad Laboratories, Hercules, CA, USA).

2.3.5 Reverse transcription polymerase chain reaction (RT-PCR)

For each target gene, published primers were used where possible (Table 1). When published primers were not available, primers were created using LightCycler Probe Design Software 2.0 (Idaho Technology, Salt Lake City, UT, USA) using accession numbers from GenBank, and the genetic sequence database of the US National Institute of Health (<http://www.ncbi.nlm.nih.gov/sites/>) (Table 2.1). The default parameters of the program were used according the manufacturer's instructions

Table 5 Primer sequence for ovine, GAPDH, β -Actin , collagen, and collagenase genes

Primer name	Sequence (5'-3')	(bp) ^a	Tm (°C)	Ta (°C)	Accession number ^b	Reference
Housekeeping gene: Glyceraldhyde-3-phosphate dehydrogenase						
GAPDH F	GGCGTGAACCACGAGAAAGTATAA	120	70	54	AF030943	(Budhia, Haring, McConnell, & Blacklaws, 2006)
GAPDH R	CCCTCCACGATGCCAAAGT					
Housekeeping gene: Beta actin						
β -Actin F	TGGCATCGTCATGGACT	193	60	55	AF129298	LightCycler Probe Design Software 2.0 ^c
β -Actin R	CTTGATGTCACGGCACAATTT					
Bone: Collagen type I						
Col1 F	ATCTGCGTCTGCCACAA	155	50	55	AF129287	LightCycler Probe Design Software 2.0 ^c
Col1 R	TTCTTGGTGACCTCAGCT					
Articular cartilage: Collagen type II						
Col2 F	ACGGTGGACGAGGTCTGACT	141	70	65	AF138883	(Young et al., 2005)
Col2 R	GGCCTGTCTCTCCACGTTCA					
Collagenase gene: Matrix metalloproteinase 2						
MMP2 F	GGCATCTCTCAGATCCGT	159	60	55	AF267159	LightCycler Probe Design Software 2.0 ^c
MMP2 R	TGCTTCTGGGTGTCCTC					

F: forward primer; R: reverse primer. ^a Amplicon length in base pairs. ^b GenBank accession number of ovine cDNA. Available online at <http://www.ncbi.nlm.nih.gov/>. ^c Idaho Technology, Salt Lake City, UT, USA.

To determine if the RNA extracted provided a suitable template for RT-PCR, 1µg of total RNA underwent RT-PCR using the AccessQuick RT-PCR System (Promega, WI, USA) as per the manufacturer's instructions incorporating specific primer sets for each target gene. RT-PCR was carried out in the PX2 Thermal Cycler (Roche Molecular Systems, Pleasanton, CA, USA) with the following cycling parameters: 45°C for 45mins, 94°C for 2mins, followed by 30 cycles of denaturation at 94°C for 30s, annealing at variable primer specific temperatures (T_a) (Table 2.1) for 1min, extension at 68°C for 2mins, and a final extension at 68°C for 7mins on completion of the cycles. Samples were then held at 4°C. Cycle optimization was performed for each primer set using GAPDH and β -actin (Appendix 5.1) as internal controls and optimal annealing temperature and number of cycles was recognised by distinct clear bands of the expected base size. In all reactions, negative controls were used to confirm the absence of RT-PCR contaminants. Ovine liver tissue was used as a positive control to confirm primer amplification of RT-PCR products. All samples underwent RT-PCR at the same time to avoid potential variations in experimental protocols.

PCR purification using Invitrogen PureLink™ PCR purification kit (Invitrogen, Auckland, New Zealand) was carried out on the initial RT-PCR target gene amplicons and confirmed by sequencing using the automatic dye-terminator cyclor sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 DNA Analyzer (Applied Biosystems Inc, CA, USA). Sequencing results were submitted to NCBI BLAST searches (<http://www.ncbi.nlm.nih.gov/sites/>) to confirm correct target amplification.

2.3.6 RT-PCR amplicon quantification

The amplified products were electrophoresed on 1%(w/v) agarose gels, stained with ethidium bromide and imaged using a Gel Doc 2000 and Quantity One imaging and analyzing 1-D software (BioRad Laboratories, 2003). Sample loadings were normalized to the housekeeping genes GAPDH and β -Actin to

permit semiquantitative comparisons in total RNA levels as previously described by Thellin *et. al.* (1999).

2.3.7 Statistical analysis

Data was analyzed using a one-way ANOVA within a Statistical Analysis System (SAS Institute Inc, Maryland, 2002). Significant differences were determined using the Dunnett's test for comparison with control and using the Tukey-Kramer test for multiple comparisons. Statistical difference was compared at $P < 0.05$.

2.4. Results

2.4.1 Comparison between tissue homogenization methods

Comparison between effectiveness of the two mechanical disruption methods to pulverize the osteochondral tissues for total RNA extraction from juvenile and skeletally mature male sheep are shown in Table 2.2. RNA purity and quantity were analysed by UV spectrophotometry and evaluated by calculating the A260:A280 and A260:A230 ratios (Fontanesi *et al.*, 2008; Yasojima *et al.*, 2001).

Mechanical disruption by mortar and pestle or by ball bearing had no significant effect on total RNA quality extracted from AC with the RNeasy® Micro Kit™. However, increased quality and quantity of total RNA extracted from SCB was observed with the ball bearing method compared to mortar and pestle and subsequent RNeasy® Micro kit™ RNA extraction ($P > 0.05$) (Table 2) in juvenile sheep.

The ball bearing method of homogenization also had a significant effect on the quantity of total RNA extracted from AC, but was not significantly different on quality or quantity of total RNA extracted from SCB with subsequent RNA extraction using the adapted manual method ($P > 0.05$) in both juvenile and skeletally mature male sheep (Table 2.2).

Table 6 Comparison between mechanical disruption methods used to extract total RNA from osteochondral tissues from juvenile and skeletally mature sheep using a commercially available kit and adaptation of a manual method.

	16 week old Suffolk male		3 year old Suffolk male	
	Mortar & pestle	Ball bearings	Mortar and pestle	Ball bearings
ARTICULAR CARTILAGE				
<u>RNeasy® Micro Kit™</u>				
RNA quality ^a	0.3665 ± 0.1128	0.3968 ± 0.0798	-	-
DNA:Protein contamination ^b	1.4985 ± 0.2204	1.6250 ± 0.1559	-	-
RNA quantity ^c	0.0132 ± 0.0043	0.0159 ± 0.0030	-	-
RNA quantity ^d	0.1080 ± 0.0199	0.1268 ± 0.0140	-	-
<u>Manual method</u>				
RNA quality ^a	1.6977 ± 0.0606	1.7975 ± 0.0631	1.8725 ± 0.0289	1.9217 ± 0.0334
DNA:Protein contamination ^b	0.4569 ± 0.0389	0.4008 ± 0.0404	0.4050 ± 0.0328	0.3967 ± 0.0378
RNA quantity ^c	0.5177 ± 0.1348	0.6758 ± 0.14029	0.2175 ± 0.0274	0.2483 ± 0.0317
RNA quantity ^d	0.2031 ± 0.0940	0.5418 ± 0.0978*	0.3008 ± 0.0663	0.6200 ± 0.0766*
SUBCHONDRAL BONE				
<u>RNeasy® Micro Kit™</u>				
RNA quality ^a	0.2000 ± 0.0148	0.5685 ± 0.0209*	-	-
DNA:Protein contamination ^b	1.4628 ± 0.1324	1.0400 ± 0.1872	-	-
RNA quantity ^c	0.0080 ± 0.0006	0.0224 ± 0.0008*	-	-
RNA quantity ^d	0.1450 ± 0.0150	0.0221 ± 0.0212*	-	-
<u>Manual method</u>				
RNA quality ^a	1.7650 ± 0.0704	1.7469 ± 0.0478	1.6983 ± 0.0772	1.7633 ± 0.7723
DNA:Protein contamination ^b	0.5775 ± 0.1249	0.7738 ± 0.0848	0.5583 ± 0.0639	0.5100 ± 0.0522
RNA quantity ^c	0.2308 ± 0.1158	0.6088 ± 0.0787	0.3500 ± 0.0876	0.6244 ± 0.0715*
RNA quantity ^d	0.2194 ± 0.1059	0.4769 ± 0.0720	0.5956 ± 0.2087	0.8933 ± 0.1704
LIVER				
<u>RNeasy® Micro Kit™</u>				
RNA quality ^a	Not done	2.0475 ± 0.0878	Not done	Not done
DNA:Protein contamination ^b	-	1.8975 ± 0.1565	-	-
RNA quantity ^c	-	0.3500 ± 0.6357	-	-
RNA quantity ^d	-	0.3380 ± 0.3223	-	-
<u>Manual method</u>				
RNA quality ^a	Not done	1.7600 ± 0.0487	Not done	Not done
DNA:Protein contamination ^b	-	1.1169 ± 0.0868	-	-
RNA quantity ^c	-	3.1784 ± 0.3526	-	-
RNA quantity ^d	-	2.1199 ± 0.2792	-	-

*Statistically significant values at 95% confidence intervals. ^a Determined by 260:280 optical density ratios,

^bDetermined by 260:230 optical density ratios, ^c Ug of RNA per ul of extract, ^d Ug of RNA per mg of tissue weight

2.4.2 Comparison of RNA quality between extraction methods

The data and residuals were found to be normally distributed (Figure 2.2). The adapted manual method consistently isolated high quality total RNA from AC and SCB (Table 2.2). The optical density A260:280 ratio of the total RNA isolated from AC with the manual method was between 1.6977 ± 0.060 and 1.9217 ± 0.0334 and was deemed of suitable quality for gene expression analysis (Fleige & Pfaffl, 2006; Marchuk et al., 1998). Similarly, the optical density A260:280 ratio of RNA isolated from the SCB by the manual method was between 1.6083 ± 0.0772 and 1.7650 ± 0.0704 . These results were in distinct contrast to the low quality RNA isolated with the RNeasy® Micro Kit™ from AC with optical density A260:280 ratio ranging between 0.3665 ± 0.1128 and 0.3969 ± 0.0798 , or from bone with optical density A260:280 ratio ranging between 0.2000 ± 0.0148 and 0.5685 ± 0.0209 (Table 2). Due to the greater efficiency of the manual method, this procedure became the method of choice for RNA extraction of all remaining tissues.

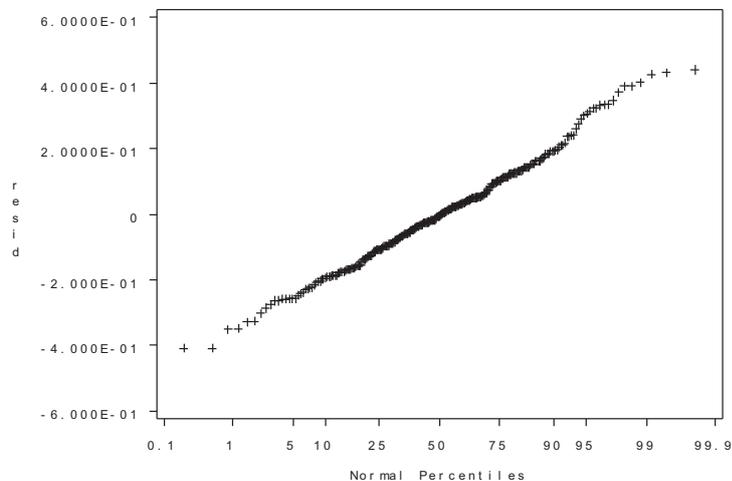


Figure 2.2 Normality plot of quality of RNA extracted from ovine liver, articular cartilage and bone.

As the quality of the RNA extracted from the osteochondral tissues from juvenile animals with the RNeasy® Micro Kit™ was insufficient for gene expression analysis (Fleige & Pfaffl, 2006) we did not pursue extraction with the RNeasy® Micro Kit™ with mature adult cartilage (Table 2.2). This is because AC in skeletally mature animals is reported to have lower mRNA levels (Young et al., 2005) compared with juvenile animals (Nixon, Brower-Toland, & Sandell, 2000).

There were differences in quality RNA extracted from the liver tissues using the RNeasy® Micro Kit™ and the manual method (Table 2.2). There were differences in quality RNA extracted from the liver tissues using the RNeasy® Micro Kit™ and the manual method (Table 2.2). The quality of RNA extracted from the liver with the RNeasy® Micro Kit™ was significantly ($P<0.05$) better with A260:A280 ratios 2.0475 ± 0.0878 compared to 1.7600 ± 0.0487 with the manual method. However, the quality of RNA extracted with the manual method was considered good quality for gene expression analysis (Adams et al., 1992). The levels of DNA:protein contamination measured as optical density A260:230 ratio were significantly ($P<0.05$) lower with the RNeasy® Micro Kit™ compared with the manual method at 1.8975 ± 0.1565 compared to 1.1169 ± 0.0868 . However, this did not interfere with RT-PCR analysis (Mortaji, Afshari, Alizadeh, & Samadi, 2008).

2.4.3 Comparison of RNA quantity between extraction methods

Residuals and the data were found to be normally distributed (Figure 2.3). The quantity of RNA extracted from the liver tissue was significantly higher with the manual method compared to the RNeasy® Micro Kit™ with 2.1199 ± 0.2792 and 0.3380 ± 0.3223 ug of RNA per mg of tissue wet weight respectively (Table 2.2).

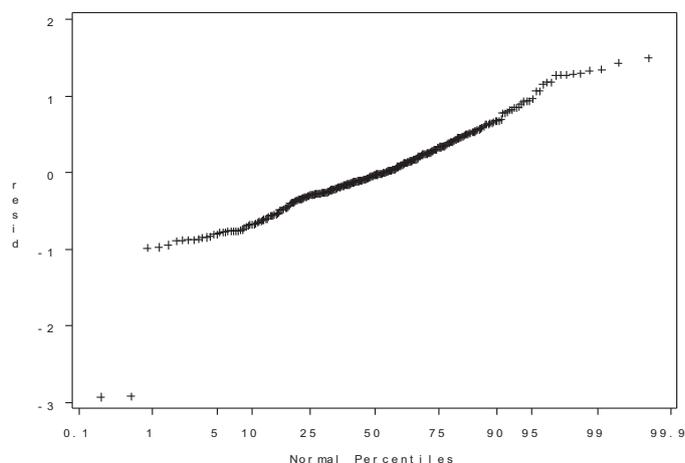


Figure 2.3 Normality plot of the quantity of RNA extracted from ovine liver, articular cartilage and bone.

2.4.4 Effectiveness of manual method to isolate RNA

Following RNA extraction from all remaining ovine fibrous and osteochondral tissues with the manual method, RNA purity and quantity were analysed by UV spectrophotometry and evaluated by calculating the A260:A280 and A260:A230 ratios (Fontanesi et al., 2008; Yasojima et al., 2001) (Table 2.3).

The purity of total RNA varied significantly between tissues with an overall optical density A260:A280 ratio mean value of 1.7000 ($P>0.0001$). Residuals and the data were found to be normally distributed (Figure 2.3). The average quality of RNA extract measured as optical density A260:A280 ratio from cellular fibrous tissues was 1.7869 ± 0.0677 . The average A260:A280 ratio of extracted RNA from the soft tissues of the synovial joint was 1.6215 ± 0.0432 . The average ratio of extracted RNA from hypocellular connective tissues was 1.7739 ± 0.0659 . Regardless of age or joint, the average quality of RNA extracted from AC or bone was 1.7490 ± 0.0667 and 1.7206 ± 0.0582 respectively (Table 2.3).

Table 7 Mean values for total RNA quality and quantity from ovine fibrous and osteochondral tissues using an adaptation of a manual RNA isolation method

	<i>DNA:Protein contamination</i>		<i>RNA quantity</i>	
	260:280	260:230	<i>Ug/ul</i>	<i>Ug/mg tissue wet weight</i>
<u>Cellular fibrous tissues</u>				
Uterine (n=2)	1.8100 ± 0.1164	0.8800 ± 0.4757	2.3000 ± 0.4135	2.3000 ± 0.4135
Liver (n=11)	1.7600 ± 0.0456	1.1169 ± 0.1866	3.1785 ± 0.1622	1.9866 ± 0.1014
Skeletal muscle (n=16)	1.7869 ± 0.0411	0.5900 ± 0.1682	1.1831 ± 0.146	0.5916 ± 0.073
<u>Connective tissues</u>				
Co-lateral ligament (n=4)	1.7350 ± 0.0822	0.4925 ± 0.3364	0.5800 ± 0.2924	0.2189 ± 0.1103
Tendon (n=4)	1.8127 ± 0.0496	1.1055 ± 0.2028	0.9300 ± 0.1763	0.5314 ± 0.1007
<u>Synovial joint</u>				
Synovial fluid (n=23)	1.6230 ± 0.0343	0.4730 ± 0.1403	1.3552 ± 0.1219	0.3115 ± 0.0280
Synovial membrane (n=10)	1.6200 ± 0.0520	0.3150 ± 0.2127	1.0140 ± 0.1849	0.5965 ± 0.1088
<u>Osteochondral tissues</u>				
<i>Articular cartilage</i>				
16 week old lamb ♂ (n=8)	1.6025 ± 0.0823	0.6550 ± 0.3364	1.2350 ± 0.2924	0.6861 ± 0.1624
1 yr old neuronalaxonal dystrophy lamb ♂ (n=8)	1.7750 ± 0.0582	0.5125 ± 0.2378	0.4050 ± 0.2067	0.0438 ± 0.2235
2 yr old ewe (n=4)	1.7250 ± 0.0823	0.3550 ± 0.3364	1.1475 ± 0.2924	1.1475 ± 0.2924
3 yr old ram (n=14)	1.8936 ± 0.0440	0.4014 ± 0.1798	0.2307 ± 0.1563	0.3076 ± 0.2084
<i>Subchondral bone</i>				
16 week old lamb ♂ (n=8)	1.8563 ± 0.0582	0.7188 ± 0.2378	0.4675 ± 0.2067	0.2597 ± 0.1148
1 yr old axonal dystrophy lamb ♂ (n=8)	1.7200 ± 0.0582	0.4450 ± 0.2378	1.0400 ± 0.2067	0.9455 ± 0.1879
2 yr old ewe (n=4)	1.7075 ± 0.0822	0.4025 ± 0.3364	1.3600 ± 0.2924	0.9714 ± 0.2089
3 yr old ram (n=14)	1.6000 ± 0.04113	0.5313 ± 0.1682	0.4938 ± 0.1462	0.9876 ± 0.0292

The total quantity of RNA extracted from each tissue varied significantly ($P < 0.0001$) using the manual method (Table 2.3). The average quantity of RNA extracted from the cellular fibrous tissues uterine and liver ($n=13$) was 2.1433 ± 0.2575 ug of RNA per mg of tissue wet weight (ug/mg), and skeletal muscle ($n=16$) 0.5916 ± 0.0730 ug/mg. From the hypocellular and dense connective tissues; collateral ligament ($n=4$) and tendon ($n=4$), between 0.2189 ± 0.1103 and 0.5314 ± 0.1007 ug/mg respectively was extracted. On average 0.4540 ± 0.0684 ug/mg was extracted from the synovial soft tissues: synovial fluid ($n=23$) and synovial membrane ($n=10$). The average quantity of RNA isolated from AC ($n=30$) and SCB ($n=34$) was 0.5463 ± 0.2217 and 0.7911 ± 0.1352 ug/mg respectively (Table 2.3).

2.4.5 Gene expression analysis

In order to determine the availability of the extracted RNA for RT-PCR and the specificity of primer sets, five genes were examined within respective gene specific tissues. PCR products for ubiquitous housekeeping genes GAPDH and β -Actin confirmed the presence of mRNA in AC, SCB, liver and uterine tissues (Figure 2.4). Successful RT-PCR amplified collagen type I products confirmed RNA was extracted from bone osteocytes (Figure 2.5). PCR products for collagen type II confirmed RNA was extracted from AC chondrocytes (Figure 2.6). PCR amplification of a proteinase gene, MMP2, confirmed RNA was extracted from uterine tissue (Figure 2.4).



Figure 2.4 Gene expression signals confirming GAPDH (120 bp), β -Actin (193 bp) and MMP2 (159 bp) in ovine liver and uterine tissue.

Key: L: 100 bp DNA ladder. Lane 1: blank, Lane 2: negative control B-Actin liver, Lane 3: B-Actin liver, Lane 4: blank, Lane 5: negative control GAPDH liver, Lane 6: GAPDH liver, Lane 7: blank, Lane 8: negative control MMP2 uterine, Lane 9: MMP2 uterine.

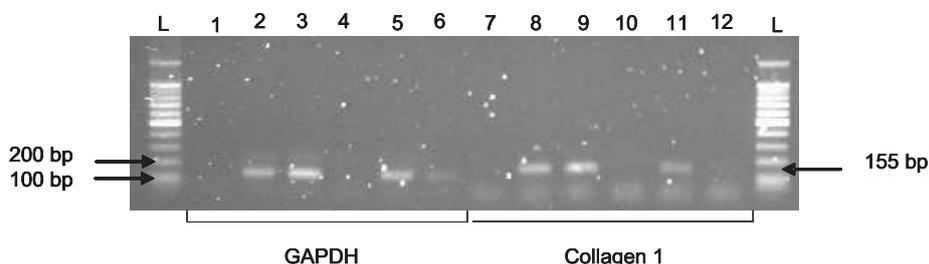


Figure 2.5 Gene expression signals for GAPDH (120 bp) and collagen type I (155 bp) for subchondral bone in skeletally mature Perendale ewes (2 years of age).

Key: L: 100bp DNA ladder. Lane 1: negative control GAPDH ewe 1, Lane 2, 3: GAPDH ewe 1, Lane 4: negative control GAPDH ewe 2, Lane 5, 6: GAPDH ewe 2, Lane 7: negative control collagen type I ewe 1, Lane 8, 9: collagen type I ewe 1, Lane 10: negative control collagen type I ewe 2, Lane 11, 12: collagen type I ewe 2.

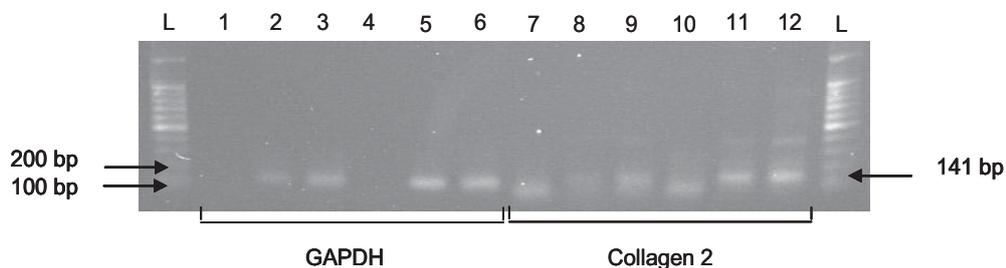


Figure 2.6 Gene expression signals for GAPDH (120 bp) and collagen type II (141 bp) for articular cartilage from skeletally mature Perendale ewes (2 years of age).

Key: L: 100bp DNA ladder. Lane 1: negative control GAPDH ewe 1, Lane 2, 3: GAPDH ewe 1, Lane 4: negative control GAPDH ewe 2, Lane 5, 6: GAPDH ewe 2, Lane 7: negative control collagen type II ewe 1, Lane 8, 9: collagen type II ewe 1, Lane 10: negative control collagen type II ewe 2, Lane 11, 12: collagen type II ewe 2.

2.5. Discussion

In this pilot study, the effectiveness of two methods of tissue disruption (mortar and pestle or ball bearings) and two methods of RNA extraction using a commercially available kit (RNeasy® Micro Kit™, Qiagen, Victoria, Australia) or an adapted manual RNA isolation method (Chomczynski & Sacchi, 1987; Heinrichs et al., 1997) were evaluated to isolate high quality RNA from ovine osteochondral and cellular tissues *in vivo*. The results demonstrated that the best quality and quantity of RNA extracted from AC, SCB and other tissues occurred with ball bearing tissue disruption followed by the adapted manual RNA extraction method.

Conversely, the quality and quantity of RNA extracted from the very small quantities of bone and cartilage with the commercially available kit were insufficient (Fleige & Pfaffl, 2006; Marchuk et al., 1998) despite these tissues being obtained from skeletally immature subjects (Adams et al., 1992; Chomczynski & Sacchi, 1987; Reno et al., 1997). This may have been related to the very small sample quantities and the dense hypocellular nature of these tissues (Adams et al., 1992; Ireland, 2003). The low yield of RNA may also have been attributed to the efficiency of the RNeasy MinElute column to remove RNA > 200 nucleotides and its capacity to act as a CsCl cushion (Adams et al., 1992). McKenna *et. al.* (2000) have reported the RNeasy Midi column (Qiagen, Victoria, Australia) became blocked following centrifugation (McKenna et al., 2000) and this may have occurred during our use of the RNeasy® Micro Kit™ (Qiagen, Victoria, Australia). It is unlikely that the low yield of RNA extracted from the hypocellular and dense osteochondral tissues was due to exceeding the maximum binding capacity of the column (45ug RNA), as significant quantities of RNA were extracted from the cellular liver samples (Table 2.3).

As RNA is susceptible to post-mortem degradation (Fontanesi et al., 2008), careful sample collection; handling and storage procedures were applied. All sample tissues were snap frozen in liquid nitrogen within 15mins of euthanasia and maintained at -80°C until RNA extraction (Fontanesi et al., 2008; McKenna et al., 2000). This was to limit RNase activity and decrease the likelihood of

poor quality RNA which is the limiting factor in gene expression study (Copoio et al., 2007). In addition, the purity and integrity of RNA is important to the outcomes of RNA based analyses (Fleige & Pfaffl, 2006) and low quality RNA preparations of an optical density of less than 1.4 are not suggested for use in RT-PCR reactions due to the likelihood of contamination with matrix proteins (Marchuk et al., 1998).

In this investigation, optical density 260:280 values (Clements et al., 2006) from RNA extracted with the manual method ranged between 1.6 – 2.0 (Table 2.3) indicating low level protein contamination (Mortaji et al., 2008). High levels of DNA:protein contamination have been reported following chloroform-phenol extraction (Chomczynski & Sacchi, 1987) and can interfere with RT-PCR. The optical density 260:230 ratio values from RNA extracted with the manual method ranged between 0.3150 – 1.1169 and indicate contamination with peptides, phenols, aromatic compounds or carbohydrates (Mortaji et al., 2008; Pinto, Thapper, Sontheim, & Lindblad, 2009). However, this did not interfere with RT-PCR analysis and we observed consistent levels of GAPDH gene expression in all tissues examined (Mortaji et al., 2008; Pinto et al., 2009; Salzman, Fujita, Zhu-Salzman, Hasegawa, & Bressan, 1999).

The expression of collagen type I and collagen type II confirmed successful RNA extraction from bone osteocytes and AC chondrocytes respectively (Figures 2.5, 2.6) (Miosge et al., 2004; Sandberg & Vuorio, 1987). The expression of MMP2 confirmed we had extracted RNA from uterine tissue (Figure 2.4) which was collected postpartum during the involutory stage (Ricke, Smith, & Smith, 2002). These results indicate that the purified RNA was predominantly free from PGs and RT-PCR inhibitors and was highly suitable for gene expression analysis (Yasojima et al., 2001).

The efficiency of the manual method to isolate RNA from ovine juvenile and adult tissue (Appendix 7.1) was high; given the very small quantity of starting tissue in wet weight (10-60mg) and the consistent RNA yield between 2-0.5ug/mg of tissue (Table 2). Previous studies have successfully isolated RNA from AC *in situ* from a variety of species including human (Adams et al.,

1992; McKenna et al., 2000), rabbit (Heinrichs et al., 1997; Reno et al., 1997), dogs (Matyas et al., 2002), sheep (Kleemann, Krockner, Cedraro, Tuischer, & Duda, 2005) and horses (MacLeod et al., 1996) (Table 2.4). However, comparison between studies must take into consideration the use of greater starting quantities of biological tissue, overnight protein digestion, extensive centrifugation steps, or cell culture. In addition, significant differences exist in cellularity and cellular metabolism in AC as well as between human and animal tissues (McKenna et al., 2000). In this investigation the quantities of RNA isolated with the manual method compared favorably with previously published yields from ovine and dense hypocellular connective tissues (Reno et al., 1997; Young et al., 2005)

The mRNA content of tissues and cells is context dependent and the levels of specific mRNA can vary physiologically with developmental age (Nixon et al., 2000) and pathology (Clements et al., 2006). The total quantity of RNA extracted from AC taken from the joints of aged 3 year old ram was significantly less than that of skeletally immature animals (Table 2.3). Age dependent changes in AC are associated with a decrease in viable cell numbers (Lindner, 1972; McKenna et al., 2000; Young et al., 2005). Similarly, the quantity of RNA extracted from cartilage samples from the 1 year old axonal dystrophy ram was significantly less than the RNA extracted from the cartilage of healthy 2 year old ewes. RNA extracted from diseased tissue can be of lower quantity and quality (Clements et al., 2006; McKenna et al., 2000). The high levels of RNA extracted from the liver, uterine and skeletal muscle are consistent with the increased cellularity of these tissues (Reno et al., 1997).

With some tissues, such as tendon, collateral ligament and synovial tissues, the total number of samples used for extraction was low. This was due to several factors including post-mortem tissue stability (Yasojima et al., 2001) and the priority to collect AC and SCB tissues. Ambient room temperature and time of year had a significant effect on tissue harvest from synovial joints. The need to keep joint tissues snap frozen with liquid nitrogen to ensure RNA stability (Fontanesi et al., 2008; McKenna et al., 2000) limited the opportunity to collect increasing number of samples of tendon, collateral ligament, synovial fluid and

synovial membrane. Some samples were lost during tissue harvest and some samples were lost during RNA extraction due to environmental conditions or operator error.

RNA analysis is a powerful tool in the determination of tissue specific gene expression during development or disease and in response to biological stimuli. Molecular techniques which measure RNA gene expression in cells or tissues include in situ hybridization, northern blot, dot blot, RNase protection assay, RNA mapping, micro array, and S1 nuclease assay (Fleige & Pfaffl, 2006; Serazin-Leroy, Denis-Henriot, Morot, de Mazancourt, & Giudicelli, 1998). However, RT-PCR is the most sensitive, specific and adaptable method able to detect extremely low levels of mRNA expression as seen with cytokines (Yin et al., 2001). High quality intact RNA is prerequisite for gene expression analysis (Copoio et al., 2007).

2.6 Conclusions

The most widely used method for total RNA isolation is the guanidinium isothiocyanate procedure (Chomczynski & Sacchi, 1987). However, contamination of RNA with highly electronegative PGs can adversely affect gel electrophoresis and PCR amplification. Modification of this method has been reported in attempt to minimise sample loss, increase the quality and quantity of RNA and suitability for gene expression analysis (Adams et al., 1992; Burton-Wurster et al., 2005; Heinrichs et al., 1997; MacLeod et al., 1996).

We have further modified these procedures and developed an RNA isolation protocol which can be used to reliably and consistently isolate high quality RNA from very small quantities of ovine bone and AC within a relatively short time. The quality and integrity of the RNA determined by optical density, gel electrophoresis, and RT-PCR normalized to ovine specific housekeeping genes GAPDH and β -Actin, reveal the RNA product to be free of RT-PCR inhibitors (Adams et al., 1992; Clements et al., 2006; Mortaji et al., 2008). A number of factors were critical to ensure successful isolation including effective tissue homogenization which incorporated mechanical and chemical tissue disruption within a single step, and protein digestion within an RNase free environment.

Table 8 Effectiveness of RNA extraction methods reported in literature

Authors	Species & tissue	Disruption & homogenization method	Total RNA extraction method	Yield ug/g tissue	A260:A280
Chomczynski & Sacchi (1987)	Rat & human (ages unknown): mammary, liver & human bone myoblast cells	Glass Teflon homogenizer, 4M GITC	Phenol:chloroform, with/without CsCl cushion centrifugation	< 0.002	< 2
Adams et al (1992)	Adult canine: shoulder & stifle joint, pooled cartilage with scalpel blade	Spex Freezer Mill 10-90 secs, 4M GITC, passed lysate through 18 gauge needle	Phenol:chloroform, ultra centrifugation CsTFA salt	< 53	< 2
MacLeod et al. (1996)	Juvenile horse, adult dog & rabbit: liver, articular cartilage pooled from: shoulder hip, elbow, stifle, cell culture, blood	Spex Freezer Mill, 4M GITC	Phenol:chloroform & RNeasy Spin Column	ND	ND
Heinrichs et al. (1997)	Juvenile NZ white rabbit: liver, kidney, muscle, growth plate tissue	Omitted pulverization step, straight to homogenization, 4M GITC	Phenol:chloroform, LiCl centrifugation, included proteinase K digestion	ND	ND
Reno et al. (1997)	Adult NZ white rabbit: cartilage, liver, kidney, lung, brain, skin, spleen, ligament, tendon	Braun Midro_Dismembrator Vessel, GITC based	TRIspin method: TRIZol Reagent & RNeasy® Total RNA Kit*	43-100	< 2.2
McKenna et al. (2000)	Adult human: pooled from knee joint	Spex Freezer Mill 10 mins	Qiagen RNeasy Midi columns*	6-8	< 2
Nixon et al. (2000)	Juvenile and adult horses: articular cartilage pooled, tendon, spleen, brain, kidney, liver, platelets, cell culture	Spex Freezer Mill, 4M GITC	Phenol:chloroform, RNeasy Mini Kit*, extensive centrifugation & repeated extraction	ND	ND
Burton-Wurster et al. (2005)	Adult canine: articular cartilage pooled from shoulder joint, cell culture	Omitted-went to directly to homogenization, 4M GITC	Digested with RNase free DNase while bound to RNeasy columns*	400-800 ng	ND
Young et al. (2005)	Adult ovine: articular cartilage pooled from stifle joint	Mikro-Dismembrator, TRIZol Reagent	RNeasy Mini Kit*, on column DNase digestion *	0.1-- 10	ND

ND: not determined, *Qiagen

Chapter 3

The effects of exercise on gene expression in articular cartilage and subchondral bone

Pedley, R.M., Howe, L., Rogers, C.

3.1 Abstract

Traumatically induced joint disease is a widespread affliction in performance horses and a major cause of loss and limitation within the equine industry. Injury to osteochondral tissues (bone, tendon, cartilage) has been related to progressive micro-injury (Kawcak et al., 2000; Nunamaker et al., 1990), chronic fatigue and the repetitive cyclic mechanical load of racing and training (Kawcak et al., 2001) during growth and development (Firth, 2006; Firth, Rogers, & Anderson, 2004). Cytokines, inflammatory mediators and metalloproteinases have been identified in the progression of joint disease (Schlueter & Orth, 2004). The aim of this study was to examine the expression of these inflammatory cytokines as well as various collagen and collagenase genes in AC and SCB of young treadmill-exercised versus unexercised pasture maintained sheep. We observed significant differences ($P<0.05$) in gene

expression between the exercised trained and the control sheep not subjected to a regular training interval. In the control sheep there were significant increases in inflammatory cytokine IL-1 β , MMP2, and collagen types I and II in the AC and SCB in response to exercise challenge. The results of this research are consistent with clinical imaging and microscopy studies which suggest that moderate exercise during early life can stimulate an adaptive response in AC and SCB (Brama, Tekoppele, Bank, Barneveld, Firth, et al., 2000; Firth, 2006; Firth & Rogers, 2005a; Lammi et al., 1993). These changes can have a chondro-protective effect (Jones et al., 2003; Otterness et al., 1998) and may reduce susceptibility to athletic injury in later life. Future research using fluorescent probes and PCR may permit quantification of gene expression in *real time* to determine the anabolic and catabolic response of AC with developmental age and exercise *in vivo*.

3.2 Introduction

Osteoarthritis is the most common DJD affecting terrestrial vertebrates (Bhosale & Richards, 2008) and the most frequent cause of lameness in horses (Schlueter & Orth, 2004). It is a heterogeneous group of conditions that culminate in defective AC and bone loss characterised by slow and progressive cartilage deterioration, SCB remodeling, inflammation of the synovium, pain and increasing disability (Orth & Schlueter, 2004; Pelletier et al., 2001).

The etiology of osteoarthritis is multifactorial involving a complex interaction of inflammatory, metabolic and mechanical signals.. It is widely held that once the integrity of AC in the superficial zone is compromised, the underlying cartilage is subjected to abnormally high strain (Arokoski et al., 2000). Disorganization of the collagen type II network and cartilage softening are early indicators of pathology (Hollander et al., 1995).

The homeostatic balance in cartilage is affected by age and regulated by several factors produced by the synovium and chondrocytes including: cytokines, inflammatory mediators and matrix metalloproteinases (Brandt,

1997; M. B. Goldring, 2000a; Hollander et al., 1995; Johnston, 1997; Schlueter & Orth, 2004; Smith, 1999; R. J. Todhunter, 1996). In normal cartilage, the balance between matrix synthesis and degradation is maintained. In early osteoarthritis, chondrocytes become hypertrophic and increase production of PGs (Appleyard et al., 1999), collagen, catabolic cytokines and matrix degrading enzymes (Hedbom & Hauselmann, 2002). Over a period of time, chondrocytes undergo dedifferentiation and an increase in matrix turnover occurs with a reduction in PG content, structure, aggregation and increase in water content (Bhosale & Richards, 2008; McIlwraith, 1996; Moreland, 2003).

In addition, aggrecan, which binds to hyaluronic acid and provides cartilage elasticity and compressibility, is cleaved by aggrecanase (Moreland, 2003). Denaturation of the collagen type II helix and cumulative loss of cartilage tensile properties (Hollander et al., 1995) and functional matrix components result in tissue instability, chondrocyte death and cartilage loss down to SCB (Thomas, Fuller, Whittles, & Sharif, 2007; Young et al., 2005). Eventually, the composition and organization of the extracellular matrix becomes significantly altered and tissue integrity and biomechanical function are compromised (Grodzinsky et al., 2000). Ultimately, disease progression will involve all tissues surrounding the affected joint (Brandt, 2003; Oegema & Visco, 1999).

An adaptive response to exercise has been observed in AC and SCB in various animal models including the horse, dog, cow, sheep, rabbit, rodents and non-human primates (Brandt, 2003). Exercise imposed during the greatest period of skeletal growth is believed to increase maximal bone strength in the adult skeleton having protective effect against bone loss (Bass, 2000; Bass et al., 1998).

Exercise is necessary for the maintenance of healthy AC (Otterness et al., 1998) and mechanical strain within physiological range has chondroprotective effects (Galois et al., 2004). Exercise of a moderate intensity and cyclic load rate cause an increase in PG synthesis and content, and increase in stiffness of the collagen fibrillar network (Roos & Dahlberg, 2005). Joint disuse and exercise intensity that exceeds physiological constraint result in cartilage

fibrillation, pitting, fissuring and gradual deterioration of the matrix and collagen network.

Matrix metalloproteinases (MMPs) play an important role in the turnover of cartilage extracellular matrix under normal and pathological conditions (Nguyen et al., 1993). MMPs are proteinases which belong to the family of zinc dependent metalloproteinases and are classified into four subgroups based on substrate specificity: collagenases, gelatinases, stromelysins, and membrane type (MT) MMPs (Hooper, 1994; Johnson et al., 1998). Collectively these enzymes are capable of degrading the macromolecules of connective tissues. MMP2 and MMP9 are gelatinases with a wide range of substrate specificity including: gelatin, elastin fibronectin, collagen I, IV, V, VII, X, XI, laminin, aggrecan, vitronectin and link protein (McCawley & Matrisian, 2001; Tanaka et al., 2000). MMP2 has an important role in joint disease and osteoarthritis and is involved in cartilage destruction (Tanaka et al., 2000) and abnormal bone collagen metabolism (Bailey & Mansell, 1997; Mansell & Bailey, 1998). In addition, elevated MMP2 levels have been observed in bovine and equine synovial fluid during osteoarthritis (Arican et al., 2000; Clegg, Coughlan, Riggs, & Carter, 1997).

During growth and skeletal development, the process of degradation is balanced by the expression of proteinases and their biological inhibitors (TIMP) (M. B. Goldring, 2000b). In normal adult tissue, MMP activity is very low and the extracellular matrix is in a state of dynamic equilibrium. This is in contrast to the upregulated/activated state observed during physiological remodeling, inflammation (Johnson et al., 1998) or joint disease (Mansell, Tarlton, & Bailey, 1997). Proinflammatory cytokines, growth factors and extracellular matrix interactions (mechanical stress and pericellular environment) are key initiators in these processes.

Few studies have reported on the interrelationship between exercise, ageing and cartilage (Arokoski et al., 2000). This research will be the first to examine the effects of exercise on gene expression of cytokines: IL-1 β and TNF- α , matrix metalloproteinase 2 (MMP2), collagen type I and collagen type II in AC

and SCB *in vivo* in exercised and unexercised pasture maintained Perendale sheep maintained at pasture.

3.3 Materials & methods

3.3.1 Animals

Twenty four female Perendale weaned lambs born in New Zealand late 2004/ early 2005 breeding season of four months of age, were purchased and transported to Massey University, Palmerston North, New Zealand. On arrival all lambs were weighed, tagged with a unique identifier and allocated to control (unexercised) (n=12) and treatment groups (treadmill exercise) (n=12). The lambs were managed together at pasture in paddocks of less than a hectare. The diet was predominantly ryegrass/clover based pasture, with supplementary meadow hay and general Lucerne pellets during winter if required to maintain adequate condition score. The animals were approved for the study and cared for according to the Massey University Animal Ethics Committee.

3.3.2 Exercise regimen

The exercise group (n=12) underwent a program of controlled treadmill exercise superimposed on their normal paddock activity. The sheep were exercised in pairs within a customized cage on a generic equine treadmill situated adjacent to the sheep paddocks (Figure Appendix 8). The exercise program conducted 3 days per week was structured to be osteo-inductive and consisted of a brief walk and trot warm-up and then a short canter and sprint (Table 3.1). At the completion of exercise the sheep were returned to pasture. Daily exercise and health records were maintained for each sheep throughout the trial.

Table 9 **Osteo-inductive exercise regimen**

Structure	Phase	Speed (m/s)	Time (seconds)
Warm-up	Walk	1.10	30
	Trot	1.85	30
Osteo-inductive	Canter	3.25	60
	Sprint	4.05	60
	Canter	3.25	60
Cool down	Trot	1.85	30
	Walk	1.10	30

3.3.3 Timeline

The first exercise program began during February 2005 when the lambs were four months old and continued for eight consecutive months until September 2005. At the conclusion of the first regime, at 12 months of age, six unexercised and six exercised lambs were euthanized and tissue collected. The remaining exercised sheep (n=6) were then spelled at pasture for the following eight months. The second and final exercise program was started during May 2006 and continued for five months until September 2006. At the final exercise treatment, all sheep (both exercise and unexercised groups) were subjected to a gallop challenge, after which the remaining unexercised (n=6) and exercised (n=6) sheep were euthanized and tissue harvested for gene expression analysis (Figure 3.1).

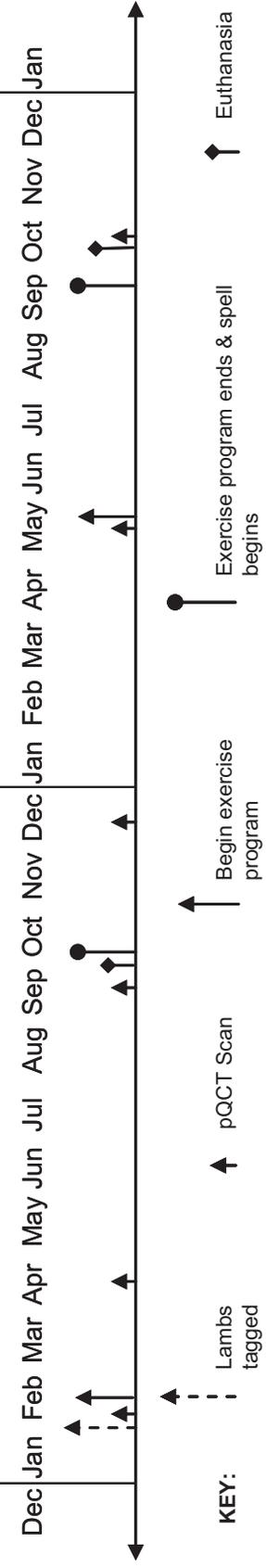


Figure 3.1 Osteo-inductive exercise training program timeline 2005-2004.

Twenty four Perendale weaned lambs born in the New Zealand 2004/2005 breeding season were purchased and transported to Massey University, Turitea, Palmerston North, on 31 January 2005. On arrival all lambs were weighed, tagged with unique identifiers and allocated to treatment groups (treadmill exercise) and control groups (unexercised) matched for sex and weight. The exercise group underwent a program of controlled exercise superimposed on their normal paddock activity. The osteo-inductive exercise program was conducted 3 days per week commencing February 17 2005. The exercise group was trained until 1 October 2005. At 12 months of age, 12 sheep (6 from each group) were euthanized and tissue collected. Between 1 October 2005 and 14 May 2006 the sheep were spelled at pasture. Exercise was reintroduced from May 15 2006 until 21 September 2006. At the final exercise all sheep were subjected to gallop challenge. All remaining sheep were euthanized in October and tissue harvested. To quantify the response of bone to the exercise program the sheep were subjected to peripheral quantitative computer tomography (pQCT) of the distal left hind leg prior to initiation of the exercise program, at regular interval throughout the trial and at completion of the exercise study.

3.3.4 Tomography scan

Prior to the start of the trial, all unexercised (n=12) and exercised (n=12) sheep were subjected to a base line peripheral quantitative computed tomography (pQCT) scan of the distal left hind leg. Further pQCT scans were done at regular interval through the trial exercise trial. For completeness this has been illustrated in Figure 3.1.

3.3.5 Tissue preparation

Within 15 minutes of euthanasia, soft tissue dissection was performed. Tissues were collected from the liver, uterus, and tarsal joint of the left hind leg using aseptic technique, rinsed in sterile RNase free phosphate buffered saline (Sigma, St Lewis, MO, USA) to remove adherent blood and blotted on filter paper (Clements et al., 2006). The skin was removed from the limb tissues. All tissue samples were snap frozen in liquid nitrogen and kept on ice for less than 60 minutes until transfer into -80°C freezer to ensure RNA stability in dense connective tissues and cellular tissues post-mortem (Fontanesi et al., 2008; Marchuk et al., 1998). All tissues remained at -80° until required for RNA extraction (Yasojima et al., 2001).

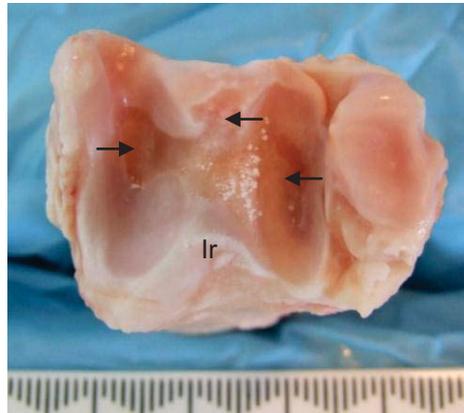
3.3.6 Limb dissection

The frozen and intact tarsal joints from skeletally mature exercised and unexercised female sheep (exercised n=7, unexercised n=6) were removed from -80°C and maintained on ice for 45-60 minutes. The frozen samples were immobilized in a small bench top vice. Gross lateral dissection through the tendons of mm. gastrocnemius et flexor digitorum superficial, m. flexor digitorum profundus, m. extensor digitorum lateralis and m. peroneus tertius were performed with a number 11 scalpel blade. Severance of the origin of the peroneus tertius, medial digital extensor and long digital extensor muscles allowed exposure of the synovial joint (Figure 3. 2).

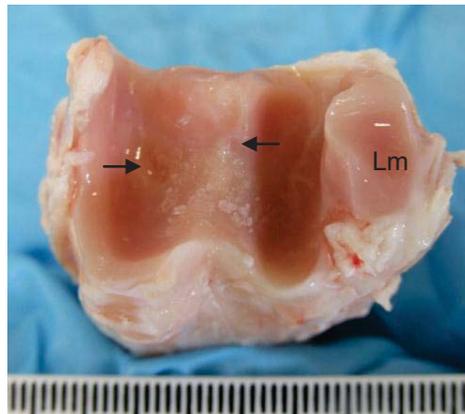
Figure 3.2 The distal tibia from exercise trained and unexercised normal two year old ewes.

a). The articular surface of the DT from an untrained control ewe. There is wide spread and severe cartilage erosion within all compartments of the joint (arrows). In particular, deep fissures within the medial trochlea groove (Mtg) extend over the intermediate ridge (Ir). b). The articular surface of an exercise trained animal. Arrows indicate areas of cartilage erosion which is less severe than that of the untrained control animal (a). The cartilage lesion extends from the medial trochlea groove over the intermediate ridge but with less severity. c). The articular surface of an exercise trained animal. There are no obvious signs of cartilage wear or degeneration within any compartments of the joint.

a. Untrained control (severe erosion)



b. Exercise trained (moderate erosion)



c. Exercise trained (no obvious erosion)



Articular cartilage and SCB samples (5-50 mg) were harvested from the intermediate ridge of the distal tibia with a sterile number 11 scalpel blade. All AC and SCB samples were wet weighed and placed into sterile 1.5 ml eppendorf tubes and maintained frozen in liquid nitrogen. Eppendorf tubes were sealed and kept on ice, placed temporarily into -20°C freezer (less than 4 hours) (Yasojima et al., 2001), then transported on ice to be stored at -80 °C until required for RNA extraction (Marchuk et al., 1998; Yasojima et al., 2001).

3.3.7 RNA Extraction

Total RNA was prepared as previously described in chapter 2 using adaptation of a manual method of RNA isolation (Chomczynski & Sacchi, 1987; Heinrichs et al., 1997) by homogenization in a guanidinium isothiocyanate solution (GITC) (Sigma, St Lewis, MO, USA), followed by phenol (USCB Corp., Cleveland, Ohio, USA) chloroform (Sigma, St Lewis, MO, USA) extraction and 2-propanol precipitation (Sigma, St Lewis, MO, USA). Any RNA preparations yielding 260:280 ratios below 1.4 were not used as previous findings indicated that samples were likely contaminated with matrix proteins which could interfere with subsequent analysis (Marchuk *et. al.*, 1997).

3.3.8 RNA analysis (RT-PCR)

.3.8 RNA Analysis (RT-PCR)

Extracted total RNA (1 ug) from each tissue was subjected to RT-PCR using the AccessQuick RT-PCR System (Promega, WI, USA) incorporating specific primer sets (Invitrogen, New Zealand Limited) as per the manufacturer's instructions. For each target gene, published ovine primer sets were used or primers were generated using the default parameters of the LightCycler Probe Design Software 2.0 (Idaho Technology, Salt Lake City, UT, USA) using accession numbers from GenBank and the genetic sequence database of the US National Institute of Health (<http://www.ncbi.nlm.nih.gov/sites/>) (Table 2).

RT-PCR was carried out in the PX2 Thermal Cycler (Roche Molecular Systems, Pleasanton, CA, USA) at cycling parameters: 45°C for 45 minutes, 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at variable primer specific temperatures (Table 2) for 1

minute, extension at 68°C for 2 minutes, and further extension at 68°C for 7 minutes on completion of the cycles. Samples were then held at 4°C. Cycle optimization was performed for each primer set. All samples underwent reverse transcription and cDNA amplification at the same time to avoid potential variations in experimental protocols. To confirm successful amplification 5ul of the final PCR product was run on a 1% agarose gel containing ethidium bromide and imaged using a Gel Doc 2000 and Quantity One imaging and analyzing 1-D software (BioRad Laboratories, 2003). Sample loadings were normalized to the housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β -Actin (beta-actin) to permit semi-quantitative comparisons in total RNA levels as previously described (Thellin et al., 1999). The relative amount of mRNA in each band was calculated using the equation (Yasojima et al., 2001): $(I_M - I_B) \times A$. Where I_M equals mean intensity of each band; I_B equals intensity of the background; A equals area of each band.

Initial positive PCR amplicons for each gene product were purified using a PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, Foster City, California, USA) to confirm genomic sequence. Successful sequencing results were compared by NCBI Blast to those other published sequences available from GenBank.

3.3.9 Statistical analysis

Significant differences were determined using the Students T test, Dunnett's test for comparison with control and using the Tukey-Kramer test for multiple comparisons. All data was examined using SAS (SAS Institute Inc, Maryland, 2002) with a significance level of $P < 0.05$.

3.4 Results

3.4.1 Gross morphology

At gross dissection, cartilage erosion, pitting and fibrillation was evident in the joints of 5/5 (100%) control animals (n=5) (Figure 3.2). AC damage was also observed in 85% (6/7) of animals within the exercised treatment (n=7) group except for one animal where there was no observable cartilage damage.

3.4.2 Gene expression

Due to logistical constraints gene expression analysis was carried out on three randomly selected control animals and three randomly selected exercised experimental animals.

a) Collagen types I and II

A 1.27 – 1.33 times greater ($P<0.05$) increase in collagen type I gene expression was observed in the AC of the unexercised control animals compared to no expression of collagen type I in the AC of the exercised animals (Figure 3.3, Appendix 9).

The expression of collagen type I in SCB was evident in all of the SCB samples taken from the unexercised control animals and the exercised animals and confirms that RNA was extracted from SCB (Appendix 10). However, the intensity of collagen type I gene expression was increased in the bone of the unexercised sheep between 1.26 – 1.32 ($P>0.05$) times compared to the level of collagen type I gene observed in the SCB of the exercised animals (Figure 3.3).

The expression of collagen type II was evident in all of the AC samples taken from the unexercised control animals and the exercised animals and confirms that RNA was extracted from AC (Appendix 11). However, the intensity of collagen type II gene expression in the AC of the unexercised control animals was 1.18 – 1.57 times greater ($P<0.05$) than the intensity of collagen type II gene expression observed in the exercised animals (Figure. 3.3,).

The expression of collagen type II gene was evident in the SCB of the unexercised control animals and was between 1.18 and 2.30 times greater ($P<0.05$) than no discernible level of collagen type II gene expression in the SCB of the exercised animals (Figure 3.3, Appendix 12).

b) Cytokine IL-1 β and TNF- α

The expression of IL-1 β was observed in the AC of all cartilage samples irrespective of exercise treatment (Figure Appendix 13). However, the intensity of IL-1 β expression in the unexercised control animals was 1.18 – 1.50 times greater ($P<0.05$) than that of the exercise trained animals (Figure 3.3, Appendix 13.1). A similar response was observed in the SCB of the same animals with IL-1 β gene expression 1.10 – 1.82 times greater ($P<0.05$) in the untrained control animals (Figure 3.3, Appendix 14).

Tumour necrosis factor alpha gene expression was barely discernable in the AC of the unexercised control animals (Figure 3.3). The expression of TNF- α was not discernable in AC of the experimental animals ($P<0.05$) (Appendix 15) and was not expressed in the SCB in any of the animals sampled (Figure 3.3, Appendix 16).

c) Matrix metalloproteinase (MMP-2)

MMP2 gene expression was observed in AC and SCB (Appendix 17, Appendix 18 respectively). The intensity of MMP2 gene expression in the AC of the untrained control sheep was increased between 1.11 and 1.77 times compared to the exercise trained animals ($P<0.05$). The expression of MMP2 in the SCB of the untrained control sheep was 1.17 – 1.38 times greater than significantly ($P<0.05$) low or no MMP2 observed in the SCB of the exercise trained sheep (Figure 3.3).

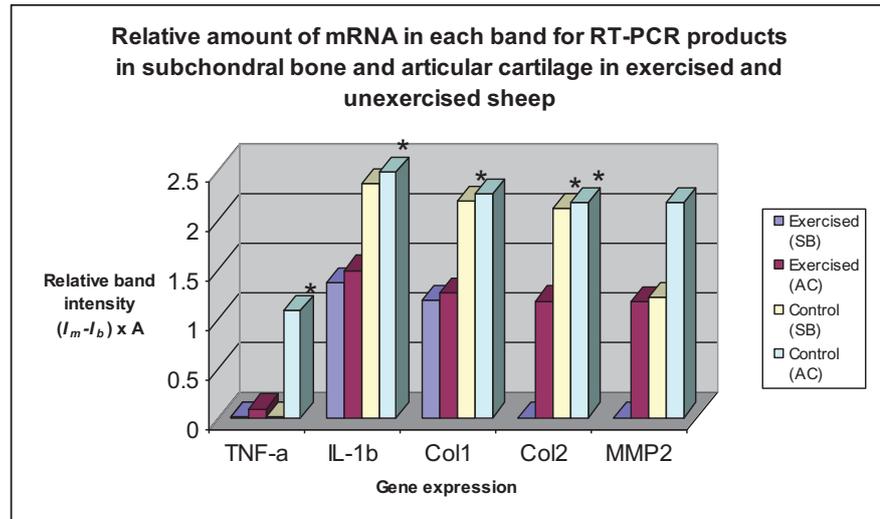


Figure 3.2 The relative amount of mRNA gene expression

The relative amounts of mRNA gene expression for inflammatory cytokines, collagen type I and collagen type II and matrix metalloproteinase 2 (MMP2) in subchondral bone from unexercised (control) and exercised (treatment) sheep. The relative amount of mRNA in each band was calculated using the equation $(I_M - I_B) \times A$. Where I_M equals mean intensity of each band; I_B equals intensity of the background; A equals area of each band, significant differences * ($P < 0.05$).

3.5 Discussion

This present study extends the work presented in chapter 2 by examining the gene expression of a number of important extracellular matrix components at the mRNA level. The aim of this study was to undertake regional assessment of gene expression in AC and SCB in unexercised and exercised pasture maintained sheep. We observed significant differences in gene expression between the two groups, with an increase in inflammatory mediator, collagenase and collagen genes occurring in the unexercised sheep compared to the exercise trained sheep.

One of the difficulties we encountered with the dense and hypocellular connective tissues was the relatively low average yield of RNA (2.85-7.058 ug/100mg) which resulted in the exclusion of some samples. The low RNA yield in this study may be attributed to the mature age of the sheep, tissue pathology and the very small samples, rather than degradation and technical factors. Cartilage activity and turnover activity decrease rapidly with age, Nixon *et. al.* (2002) and Young (2005) reported low levels of mRNA in cartilage from skeletally mature adult tissue compared to cartilage from skeletally immature juvenile tissue. Lower yields of RNA have been reported from osteoarthritic (0.669 ug/100mg) and normal adult AC (0.839 ug/100mg) pooled from the knee joint (McKenna *et al.*, 2000). Although we were able to isolate cartilage from specific sites within the joint, the low yield of RNA precluded the examination of an increasing number of cytokine, MMP and collagen genes within this region with semi-quantitative RT-PCR.

During gross tissue dissection, we observed damage to the cartilage of the lateral, intermediate and medial regions of the distal tibia in all the animals examined except for one of the exercised animals. Consistent with this morphology were observed changes in gene expression. Although our findings are limited to a single time point, these changes were likely associated with active degradation of cartilage and SCB remodeling in response to exercise and biomechanical demand. Our results support the hypothesis that moderate exercise during the prepubertal period can stimulate an adaptive response in AC and SCB and this response may have a chondroprotective effect against high intensity exercise in later life. In contrast, unexercised pasture maintained control animals subjected to high intensity gallop exercise had an increase in inflammatory cytokines, MMP2 and collagen types I and II in AC and SCB.

Normal AC is specific for collagen type II as well as associated collagens IX and XI and does not contain collagen types I or III (Miosge *et al.*, 2004; Sandberg & Vuorio, 1987). However, chondrocytes have been reported to alter collagen phenotype *in vitro* and *in vivo* in response to specific stimuli (Achnabel *et al.*, 2002; Mackie & Ramsey, 1996). Discoordinate gene expression is a characteristic feature and diagnostic marker of chondrocyte

metabolism during early stage joint disease (Adam & Deyl, 1983; Matyas et al., 2002; Young et al., 2005).

In this present study we observed an increase in collagen type I expression in AC of the pasture maintained control sheep subjected to gallop exercise. The altered gene expression and chondrocyte phenotype are similar to that observed in human articular cartilage during development, chondroblast dedifferentiation, hypertrophy (as seen in the growth plate) or regeneration of mature cartilage (Sandell & Aigner, 2001). Bianco et. al. (1998) reported hypertrophic chondrocytes *in vitro* further differentiate to osteoblastic cells and synthesise collagen type I in a variety of animal models (Bianco et al., 1998). The substitution of collagen type II with the less glycosylated collagen type I ubiquitous to skin and bone, has been attributed to cartilage functional defect and related to the production of adhesion cell surface proteins in human adult articular cartilage (Nimni & Deshmukh, 1973).

We observed an increase in gene expression for collagen type II in AC from the pasture maintained control animals. In early osteoarthritis, a transient proliferative chondrocyte repair response occurs with increased synthesis of matrix components: collagen (Aigner, Zien, Hanisch, & Zimmer, 2003; Appleyard et al., 2003), aggrecan (Matyas, Adams, Huang, & Sandell, 1995), small leucine rich PGs (Young et al., 2005), catabolic cytokines and matrix degrading enzymes (M. B. Goldring, 2000b; S. R. Goldring & Goldring, 2004). However, the repair of matrix components is limited as the normal cartilage matrix architecture cannot be restored by the adult chondrocyte (M. B. Goldring, 2000b). As a result, the thicker and biomechanically inferior cartilage undergoes degenerative change over time. This has been attributed to loss and failure of articular chondrocytes to: replace water binding PGs (Sandell & Aigner, 2001), maintain fixed charge density, and dysregulation between the collagen network and growth factors (M. B. Goldring, 2000a, 2000b). Once damaged, adult AC has little capacity to repair itself (Barbero et al., 2004; McIlwraith, 1996).

Cheung (1978) demonstrated in juvenile rabbits that collagen type II is synthesised within hours and weeks of injury to the AC. Interestingly, no type I or III collagen was synthesised in the tissue plug filling the deep lacerations which penetrated the calcified regions. The expression of collagen type I in the cartilage of the control animals in our study may suggest that the injury to the cartilage occurred in the mature adult tissue and likely to be in response to the most recent gallop exercise.

According to Yoo et. al. (1998) the expression of collagen type II in SCB is evidence of injury and repair response. When articular cartilage injury extends beyond the SCB, pluripotential mesenchymal cells migrate into the site of injury. Yoo et. al. (1998) demonstrated *in vitro*, that these bone-marrow-derived cells can generate collagen type II (and collagen type X) within three to five days in serum-free culture conditions. In addition, immunodetection of large and small PGs and GAGs indicated the presence of cartilage PGs. Terminal differentiation of these cells into hypertrophic chondrocytes demonstrated the reestablishment of SCB in the repair of AC (Yoo et al., 1998). We observed collagen type II gene expression in the SCB of the unexercised animals subjected to the final gallop challenge but not in the exercise trained sheep. This would suggest that moderate exercise regime that sheep were subjected to had a chondroprotective effect on the AC and SCB.

Collagen type I is widespread throughout the body excluding hyaline cartilage (Viguet-Carrin et al., 2006) and is the main constituent of bone providing structural support and the transduction of mechanical stimuli (Duncan, 1995; J. Rubin, Rubin, & Jacobs, 2006). We observed an increase in collagen type I in SCB of the untrained pasture maintained sheep. Mansell and Bailey (1998) reported an increase in SCB collagen metabolism in human osteoarthritic femoral heads. The increased collagen metabolism measured indirectly as a three–four times increase in TGF β and increased MMP2 activity is a measure of the osteoarthritic potential of bone (Mansell & Bailey, 1998). However, with increased bone collagen metabolism, the newly deposited bone is

hypomineralised and this may further exacerbate cartilage deterioration by altering joint morphology and biomechanical function.

Cytokines IL-1 and TNF- α have received a great deal of attention in the pathology of joint disease. These proinflammatory mediators are produced by cells of the synovium, chondrocytes and mononuclear cells and are central mediators in catabolic metabolism and in the initiation of MMP gene expression (Hedbom & Hauselmann, 2002). TNF- α and IL-1 β can decrease chondrocyte synthetic pathways and upregulate degradative processes (McIlwraith, 1996). The results of our study are consistent with the extensive literature that documents the active role IL-1 plays in the resorption of cartilage and connective tissue. IL-1 has proven to be much more potent *in vivo* than TNF- α in early cartilage degeneration (Van Lent et al., 1995).

IL-1 β gene expression was evident in all the AC samples tested but was greater (1.1767 – 1.4952 times greater) in the AC of the control sheep. A particularly novel finding in the present study was the low level of TNF- α gene expression in these same animals and no TNF- α expression discernible in the exercised animals. Van Lent *et. al.* (1994) reported TNF- α was undetectable *in vivo* during early immune complex arthritis in mice (Van Lent et al., 1995). *In vitro*, TNF- α may cause significant PG suppression and contribute to cartilage deterioration (Tyler, 1985). However, very large quantities of TNF- α were necessary (100ng/ml).

The findings of Joosten *et. al.* (1999), argue against TNF- α -dependent IL-1 production in mice with collagen type II induced arthritis (Joosten, Helsen, Saxne, van de Loo, & van den Berg, 1999). Their results show that the inhibition of IL-1 β protects cartilage and bone from destruction, evident as reduced serum COMP levels and VDIPEN neoepitope in cartilage, a marker of MMP activity. In the exercised trained animals we observed low levels of IL-1 β expression concomitantly with very low or no level of MMP2 gene expression. The lack of discernible MMP2 gene expression in the SCB of the exercise trained animals may indicate that an adaptive response to the exercise training regimen may have already occurred in these animals.

MMPs are responsible for extracellular matrix remodeling and the activity of MMPs is tightly regulated *in vivo* (Woessner, 1991). The expression of MMP2 in AC (Blain et al., 2001) is associated with excessive matrix catabolism and the degradation of cartilage and bone (Blain et al., 2001; Brama, TeKoppele, Beekman, van Weeren, & Barneveld, 1998; Hulejová et al., 2007; Malesud, 2006a; Ortega et al., 2003; Tanaka et al., 2000). MMP2 has been observed in synovial fluid in naturally occurring joint disease in horses (Trumble et al., 2001).

Articular cartilage is an avascular tissue with restricted blood vessel invasion except during endochondral bone development (Malesud, 2006b) or during joint disease (Bonnet & Walsh, 2005; Le, Xue, Castelnoble, & Jackson, 2007). In damaged vertebral disc cartilage, activity by MMPs may loosen the cartilage matrix cell-cell interactions and favour the penetration of blood vessels and fibroblast migration to the injured site and facilitate repair (Pattison, Melrose, Ghosh, & Taylor, 2001). The increased levels of MMP2 observed in the AC of the pasture maintained control sheep may have been a repair response in the damaged cartilage tissue (Bonnet & Walsh, 2005). MMP2 plays an important role in angiogenesis and the vascularisation of growth plate cartilage (Le et al., 2007).

Consistent with increased expression of MMP2 in cartilage of the untrained pasture maintained control animals, were the elevated levels of IL-1 β (Shen, Melrose, Ghosh, & Taylor, 2003). Ito (1996) reported MMPs 1, 2, 3 and 9 secreted from human fibroblasts in cell culture, degrade IL-1 β but not IL-1 α (Ito et al., 1996). Ito et. al. (1996) concluded that MMPs induced by IL-1 β regulate the biological activities of IL-1 β during inflammation and tissue injury through negative feedback (Ito et al., 1996).

The increased expression of MMP2 in the SCB of the untrained pasture maintained sheep compared to no MMP2 expression in the SCB of the exercised animals is an indication of bone collagen remodeling (Mansell & Bailey, 1998) in response to exercise. Bone adapts rapidly in response to mechanical forces whereby physiological load stimulates bone formation in

contrast to disuse which culminates in tissue loss (J. Rubin et al., 2006). However, the occurrence of MMP2 in conjunction with cartilage erosion is strong indicator of MMP2 involvement in joint pathology (Mansell & Bailey, 1998; Mansell et al., 1997). Increased MMP2 activity has been localised in SCB and calcified cartilage in equine osteochondritis (Al-Hizab, Clegg, Thompson, & Carter, 2002) as well as in fibrocartilage of the human mandibular joint during osteoarthritis (Tanaka et al., 2000).

Exercise during skeletal development

It is difficult to determine when the growing skeleton (modeling) is most responsive to exercise due to differing temporal patterns of bone growth in size and mass and the unique endocrine response between infancy, childhood and puberty (Bass et al., 1998). Physical activity during prepubertal years is the most opportune time to accumulate bone mass (Anderson, 2000) and is attributed to the synergistic effects of growth hormone (prepubertal years) or sex steroids (peripubertal years) (Bass, 2000). In particular, high impact sports are associated with unique loading patterns the benefits of which appear to be retained in adulthood (Bass et al., 1998). However, the effects of exercise on the adult skeleton (remodeling) are not as great as that observed in the developing skeleton (Forwood, 2008; Kannus et al., 1995).

We used age, weight and gender matched Perendale sheep subjected to a controlled exercise training regimen during their greatest period of skeletal growth (Edey, Chu, Kilgour, Smith, & Tervit, 1977). Although this study lacked control animals which were not galloped, we note that Brama *et. al.* (2002) conducted a study on the development of AC from birth to maturity in unexercised and exercised pasture maintained foals (P.A.J Brama et al., 2002). Brama *et. al.* (2002) demonstrated differences between groups and concluded that functional adaptation and site specific biochemical changes in cartilage are delayed in foals deprived of exercise during early life. In addition, this delay was not compensated for after the introduction of moderate exercise indicating that a certain level of exercise is essential during early life for functional adaptation of AC (P.A.J. Brama et al., 2002).

3.6 Conclusions

This study demonstrates that regular and moderate exercise during the period of greatest skeletal growth and prior to the attainment of skeletal maturity induces changes in cartilage metabolism and SCB *in vivo* which may enable the tissue to withstand exercise challenge in later life. This has been demonstrated by the acute inflammatory response observed in the AC and SCB of the unexercised animals subjected to high intensity exercise at the conclusion of the trial. It is unknown equivocally if the cartilage damage we observed in the joints of the exercise trained animals was due to the final gallop exercise at the end of the trial or accumulative damage sustained throughout the duration of the training regimen.

Our findings support the hypothesis that collagen type I and collagen type II are involved in the deterioration of cartilage as a result of mechanical injury (Miosge et al., 2004). In this study IL-1 β not TNF- α was the main cytokine involved in cartilage deterioration *in vivo* in exercised and unexercised sheep. MMP2 was a significant factor in cartilage and bone in conjunction with an inflammatory response. However, due to the limited number of genes examined, we cannot determine whether increased bone metabolism evidenced as increased MMP2 is a result of, or preceded by, cartilage erosion (Kawcak et al., 2001). Multiple regulatory pathways by which chondrocytes receive and respond to stimuli are likely involved (Grodzinsky et al., 2000).

This study illustrates the complexity of designing training regimens which can prevent cartilage destruction in later life (van der Harst, 2005). Further *in vivo* studies are required to determine the influence of exercise on skeletal development and the interaction between biomechanical and biochemical factors.

Chapter 4

General discussion

4.1 Introduction

Osteoarthritis is a debilitating joint disease that affects animals as they age. It has a complex etiology not yet fully understood involving biomechanical and biochemical factors which culminate in AC and SCB loss and soft tissue inflammation (Ghosh & Smith, 2002). In sport and racing horses, OA affecting the proximal interphalangeal and tarsocrural joints can develop over time due to repetitive cyclic trauma, environmental factors or as naturally occurring joint disease (Cantley et al., 1999; Vanderperren et al., 2009). OA causes significant welfare and economic cost to production and performance animals (Arican et al., 2000; Jeffcott, Rosedale, et al., 1982).

The overall aims of this thesis were to establish a reliable method for RNA extraction from ovine AC and SCB and to carry out an *in vivo* investigation on the effects of exercise on gene expression in these tissues. The purpose of this was to test the hypothesis that moderate exercise during early life would have a chondroprotective effect in AC and SCB, and this would induce an adaptive response which would equip the osteochondral tissues the ability to sustain exercise challenge in later life.

We examined the expression of specific collagen, cytokine and matrix metalloproteinase genes using the ovine model as replacement for the costly horse. The results of this study revealed statistically significant differences in gene expression between the exercised trained and unexercised control sheep. Sheep that had undergone the regular training regimen had significantly lower levels of inflammatory cytokine and matrix degrading collagenase gene expression in response to the exercise challenge, compared to the unexercised control sheep. The collagen gene expression in the AC and SCB of the unexercised control sheep was consistent with the onset of early stage joint disease. The results of this study, although limited to small sample size, support the hypothesis that exercise during early life can have chondroprotective effect on AC and SCB and this can enable the tissue to withstand exercise challenge in later life as evident by gene expression analysis.

4.2 RNA Extraction method

The adapted manual RNA extraction protocol reported in Chapter 2 was effective in the isolation of RNA from the very small samples of dense and hypocellular connective tissues. Although the extraction and purification of RNA from AC is problematic (Clements et al., 2006) the results demonstrate RNA extracted by this method was of good quality and integrity, suitable for gene expression analysis (Fleige & Pfaffl, 2006). In comparison to previously published protocols, the adapted manual method yielded relatively high levels of RNA per mg of tissue wet weight from young, skeletally mature, and osteoarthritic AC, SCB, as well as parenchymal tissues (Burton-Wurster, Borden, Lust, & MacLeod, 1998; Chomczynski & Sacchi, 1987; McKenna et al., 2000; Young et al., 2005). Although high levels of contamination with matrix proteins and PGs have been reported with phenol:chloroform based RNA extraction methods (Adams et al., 1992; Chomczynski & Sacchi, 1987), we were able to reliably perform RT-PCR (Mortaji et al., 2008; Pinto et al., 2009; Yasojima et al., 2001) on tissue specific genes confirming the effectiveness of this RNA extraction method (Miosge et al., 2004; Ricke et al., 2002; Sandberg & Vuorio, 1987).

The success of the adapted manual method may be attributed to several factors. This included careful and timely sample collection to minimise RNA degradation post-mortem and the incorporation of mechanical pulverization and chemical lysis in to a single step. This minimised sample loss and maximized tissue disruption and lysis. The extended proteinase K (Sigma, St Lewis, MO, USA) digestion at 0.72 gm/liter (Heinrichs et al., 1997) for 60 mins at room temperature allowed complete disassociation of nucleoprotein complexes. However, unlike Heinrichs *et. al.* (1997), the use of 8M LiCl to precipitate out RNA at -20°C for 3 hours followed by centrifugation did not result in any appreciable yield of RNA, and was omitted from our protocol. Similarly, on column DNase digestion (Qiagen, Victoria, Australia) and RNeasy MinElute Spin column (Qiagen, Victoria, Australia) resulted in insufficient RNA for gene analysis investigation (Copoys et al., 2007) as determined by optical density A260:A280 ratio (Fleige & Pfaffl, 2006).

The extraction protocol reported here would be useful in diagnostic and prognostic analysis where limited biological sample is available. The major disadvantage of this RNA extraction method is the use of highly toxic chemical reagents: guanidium-isothiocyanate, phenol and chloroform (Mortaji et al., 2008). As a result a very high level of laboratory safety was necessary.

4.3 The effects of exercise on gene expression in articular cartilage and subchondral bone

The results reported in Chapter 3, demonstrate differences in the amounts and types of collagen, inflammatory cytokines and MMP2 genes expressed in AC and SCB in response to exercise. In the untrained pasture maintained control animals, discoordinate changes in gene expression consistent with characteristic diagnostic features of early stage joint disease are evident (Matyas et al., 1995; Matyas et al., 2002).

The gross cartilage degeneration observed in the joints of unexercised control and exercise trained animals suggests that the intensity of the gallop exercise and the osteo-inductive exercise regimen exceeded physiological capacity of

the joint surfaces and this resulted in injury. Acute mechanical loads *in vivo* are known to cause severe cartilage damage and high rates of mechanical load induce chondrocyte death and fissures *in vitro* (Ewers et al., 2001). Brama et. al. (2000) observed gross lesions, wear lines, microdamage and loosening of the collagen network in 2 year old Thoroughbred racehorses. This demonstrates the difficulty in determining the threshold at which the intensity of exercise is sufficient for adaptive change yet within physiological limitation. In addition, the window of opportunity for adaptive change in articular tissues is limited and not specifically defined in all species. What is known is that accumulative cyclic load and high levels of mechanical strain can irreversibly injure AC (Appleyard et al., 2003; Barbero et al., 2004; McIlwraith, 1996).

The reported increases of collagen types I and II gene expression in the AC of the untrained pasture maintained control sheep are consistent with pathological change (Young et al., 2005). Although, discrepancies between studies have been reported, with no significant differences in gene expression between control and osteoarthritic cartilage (I. Martin et al., 2001). This is likely related to differences in chondrocyte differentiation, variability between individuals, and limitations inherent to the sensitivity of the methodology to detect low level gene expression (I. Martin et al., 2001). Phenotypic changes in collagen metabolism are widely accepted diagnostic features of early stage joint disease and subsequent alterations to the collagen network result in a biomechanically inferior tissue (Blain et al., 2001; Nimni & Deshmukh, 1973).

In this study, IL-1 β gene expression was evident in all AC and SCB examined but with greater intensity in the untrained control animals. This finding is consistent with the gross cartilage morphology and evidence of cartilage wear and deterioration. However, the low levels of TNF- α gene expression in the same tissues was unexpected. TNF- α gene expression had been consistently observed in all tissues tested prior to the tissues of the experimental trial, and considered ubiquitous. However, joint disease is not TNF- α dependent and IL-1 β has been reported the driver of inflammation. Cytokine IL-1 can markedly

suppress PG synthesis and accelerate cartilage destruction and amplify matrix depletion (Van de Loo & Van den Berg, 1990).

The lack of MMP2 in the cartilage and bone of the exercise trained animals, suggest the gallop exercise challenge at completion of the exercise trial, did not initiate matrix remodeling in either tissue. This indicates that the training regimen during the greatest period of skeletal growth induced some adaptive response in these tissues. MMP gene expression is not detectable in normal healthy AC (Freemont et al., 1997) and although latent gelatinases are activated with mechanical load *in vitro* expression is rapidly lost once load ceases (Blain et al., 2001). In addition, MMP2 activity is higher in immature cartilage compared to that of mature subjects and for this reason, age must be considered when evaluating MMP activity in AC (Brama et al., 1998).

4.4 Limitations of this study

The main limitation of this study was the limited number of isolated RNA samples that were analysed in Chapter 3. This was related to the difficulty of extracting RNA from dense and hypocellular connective tissues, and from normal and diseased tissues. This research was a pilot study, requiring foremost the development of a reliable and consistent methodology to extract RNA from bone. Budgeting constraints limited the total number of samples that could be analysed and the number of genes that could be examined by conventional PCR. Whilst semi-quantitative PCR is very sensitive and able to detect low level gene expression, it is of limited accuracy due to detection by semi-quantitative imaging and analysis performed using a constant number of amplification cycles (Komuro et al., 2001).

At the time of the sheep exercise treatment trial, unexercised control sheep not subjected to the final gallop exercise were not reported. Whilst our data is limited to a single time point, reference is made to other studies which demonstrated significant differences between exercise trained and exercise deprived foals with adaptive response to exercise observed in the exercise trained animals (P.A.J Brama et al., 2002). In the sheep exercise trial reported

here, the total numbers of experimental animals required were kept to a minimum. However, the results of this gene expression analysis are consistent with the literature regarding early onset pathology in AC and SCB in sheep, human and other large animal models (Arican et al., 2000; Alan Boyde, 2003; Young et al., 2005).

In this study the extent of cartilage matrix damage was not correlated to chondrocyte death. Samples used for RNA analysis are highly susceptible to degradation and can only be used once. However, the degree of matrix damage related to rate of mechanical load has been reported. Chondrocyte apoptosis occurs at threshold levels below which macroscopic damage occurs (Grodzinsky et al., 2000). High rate mechanical load resulted in cell death adjacent to fissures, and low rate mechanical load related to diffuse distribution of dead cells distant from fissures (Ewers et al., 2001). In the sheep exercise trial it is likely that chondrocyte death occurred in the damaged cartilage matrix of the exercise trained and untrained sheep. Future studies may examine the location of apoptotic cells in conjunction with gene expression. This may provide insight to the intensity of strain related to a training regimen and may assist in the design of chondroprotective training programs in the future.

We cannot determine if the changes in AC preceded or followed changes in the SCB. Our data demonstrate that changes occurred in both cartilage and bone of the untrained pasture maintained control animals. Further studies are required to determine the origin and process of tissue degeneration.

4.5 Future studies

Whilst this study will be one of the first to report Perendale sheep as a model to investigate the effects of exercise on AC and SCB during skeletal development, further *in vivo* studies are required. Future studies may determine the interaction between inflammatory cytokines, proteinases and collagen components within specific joint regions, synovial tissues and supporting soft tissue structures. In addition, examining- early exercise trained subjects during later life may give greater insight to the benefits of exercise on

AC. Sheep provide an excellent model for orthopedic research due to their availability, ease of handling, low maintenance cost, and the similarities in skeletal metabolism to human and other large quadrupeds.

To further our understanding of joint disease, its prevention and therapeutic targets for intervention, quantification of gene expression in real time (qRT-PCT) is necessary. Real time PCR is the most powerful tool for quantitative gene analysis (Kubista et al., 2006). With rt-PCR, molecular events can be correlated to physiological status (Valasek & Repa, 2005) and allow direct measurement of chondrocyte metabolism and cartilage health *in vivo* (Matyas et al., 2002).

Future studies may examine different collagen types to determine physiological status. For example, Adams and Deyl (1983) explain the production of collagen types I and III in osteoarthritic cartilage as the result of attempt to repair or to vascularization based on the absence of collagen type IV in diseased tissue (Adam & Deyl, 1983). Collagen type VI is a minor protein in AC comprising less than 2% of the cartilage matrix (Eyre et al., 1990). It functions to anchor chondrocytes within the matrix acting as a 'bridging molecule' between chondrocytes and the cellular surface (Pullig, Weseloh, & Swoboda, 1999). An increase in collagen type VI has been observed in the interterritorial matrix in the middle and deep layers of osteoarthritic human cartilage (Pullig et al., 1999).

The biomechanical stimuli which initiate the hypertrophic phenotype and increase in collagenase activity is not entirely understood (Tetlow, Adlam, & Woolley, 2001). Identifying the interactions between different MMPs and their TIMPs in matrix synthesis and degradation and how these molecules interact with cytokines and matrix proteins will assist in determining chondrocyte differentiation and phenotype during growth, development, disease and in response to exercise.

Future studies may examine the role of angiogenesis or vasculogenesis in cartilage and the expression of growth factors which may ameliorate the effects

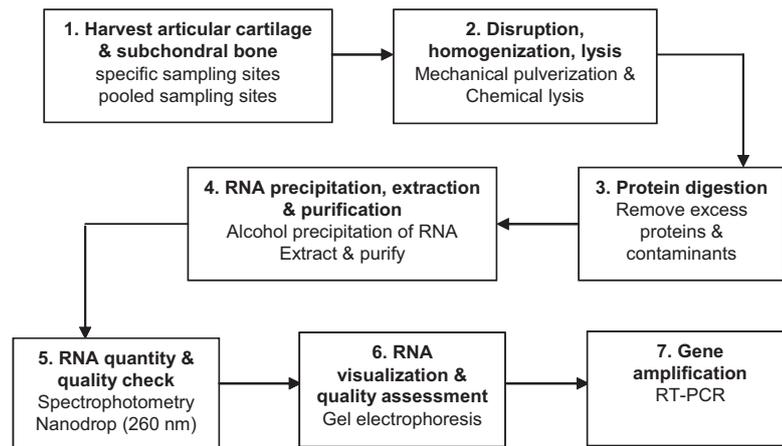
of cytokines (Ortega et al., 2003; Van Beuningen, Van der Kraan, Arntz, & van den Berg, 1993) (TGF β) and regulate cartilage (Tetlow et al., 2001) and bone remodeling and repair (Bailey & Mansell, 1997; Bonnet & Walsh, 2005). Examination of COMP levels in cartilage may provide insight into pathological tissue turnover (Joosten et al., 1999) in unexercised and exercised animals.

Immunohistochemical techniques and fluorescent microscopy would be valuable to confirm the presence of cellular receptors involved in angiogenesis or vasculogenesis and the role of VEGF in cartilage tissue. This would assist in determining the impacts of exercise during skeletal development on tissue remodeling or the formation of new vessels.

Further research at the molecular level, is necessary to determine the interactions between inflammatory mediators, matrix degrading enzymes and collagen in cartilage and bone in response to exercise during skeletal development. This will assist in the design of training regimens which optimize bone health and decrease bone injury in athletic and performance horses, and provide economic and welfare benefits for the equine industry.

APPENDICES

Appendix 1: Flow diagram of RNA extraction & gene analysis



Outline of methodology used for total RNA extraction from ovine tissue

Appendix 2: Hock joint prior to dissection

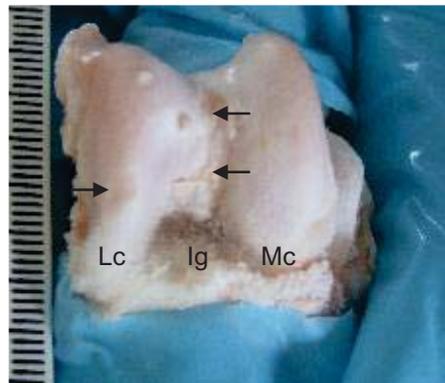
The hock joint following removal from -80°C freezer and prior to tissue dissection.



Frozen and intact sheep hock prior to tissue dissection

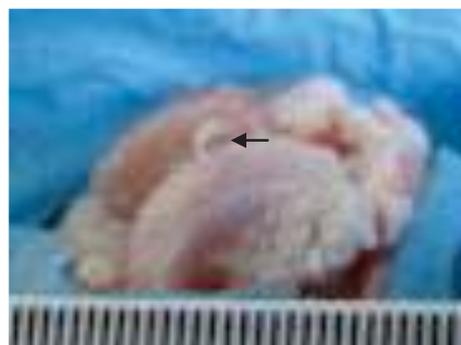
Appendix 3: The talus from an exercised ewe

The talus from an exercise trained sheep. Note the smooth glassy appearance of the hyaline cartilage and damage evident in the intermedial groove. AC was removed from the talus cochlea with a zesting tool, forming thin shavings of cartilage to be easily collected for RNA extraction and subsequent gene analysis.



The corresponding talus from exercise trained sheep (see Figure 2.1). Normal hyaline cartilage of the medial cochlea (Mc) has a smooth glassy appearance. Cartilage damage (arrows) is evident in the intermedial groove (Ig) and lateral cochlea (Lc).

Cartilage damage to the talus

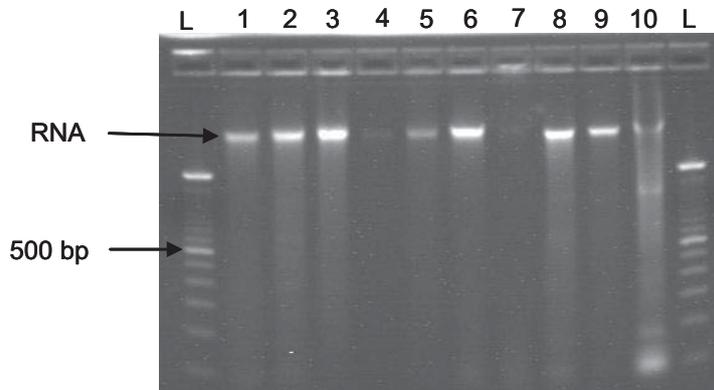


Articular cartilage was shaved from the cochlea of the talus with a zesting tool allowing thin sections of articular cartilage to be harvested for RNA extraction and gene expression analysis.

Collecting articular cartilage from the talus

Appendix 4: RNA titration

Gel electrophoresis of RNA extracted from AC and SCB isolated with the modified manual method for RNA extraction. Tissue samples are of varying size (5-40 mg). Tissue has been disrupted with either mortar and pestle (MP) or ball bearing (BB) method.

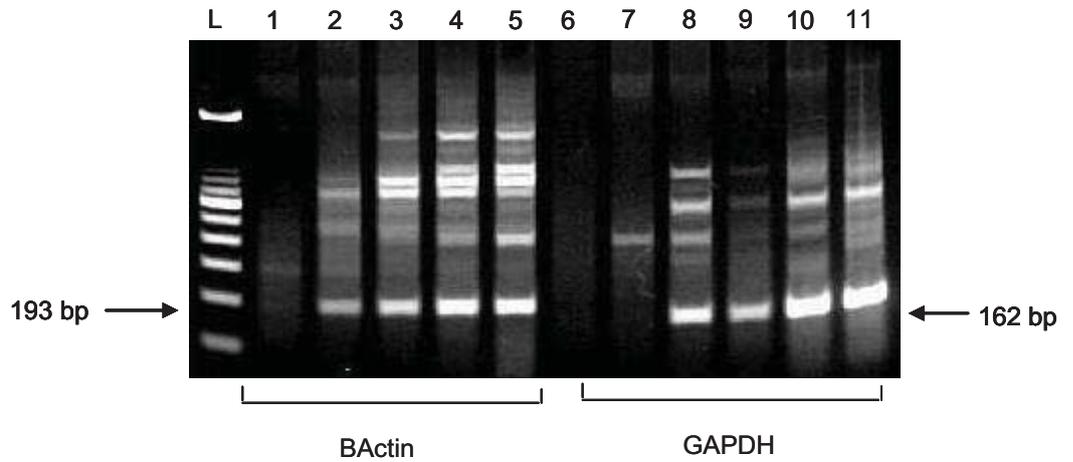


RNA titration

Lanes L are 100 bp DNA ladder, Lane 1: 15 mg AC BB, Lane 2: 15 mg SCB MP, Lane 3: 15 mg SCB BB, Lane 4: 5 mg SCB BB, Lane 5: 10 mg SCB BB, Lane 6: 40 mg AC MP, Lane 7: BLANK, Lane 8: 40 mg SCB MP, Lane 9: 40 mg SCB BB, Lane 10: 40 mg Liver BB.

Appendix 5: Optimizing MgSO₄ parameters for RT-PCR

Optimising MgSO₄ concentration for RT-PCR with β-Actin and GAPDH on ovine liver. MgSO₄ was titrated to determine the optimal concentration for gene expression of housekeeping genes β-Actin and GAPDH (1 mM).

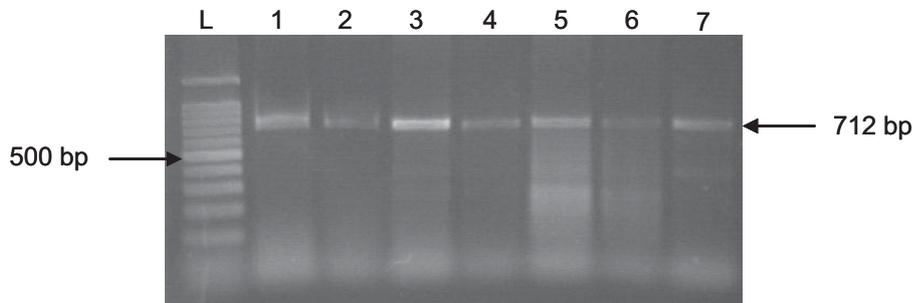


Optimising MgSO₄ for RT-PCR

Lanes L: 100 bp DNA Ladder. Lane 1: 0.5 mM, Lane 2: 0.75 mM, Lane 3: 1 mM, Lane 4: 1.50 mM, Lane 5: 2.0 mM, Lane 6: 0.25 mM, Lane 7: 0.5 mM, Lane 8: 0.75 mM, Lane 9: 1 mM, Lane 10: 1.50 mM, Lane 11: 2.0 mM.

Appendix 6: TNF- α gene expression in diseased articular tissues

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for **TNF- α** from synovial fluid, AC and SCB from a 1 year old ewe with axonal dystrophy. Sample loadings titrated at 2.5 ul and 5 ul. The expression of **TNF- α** is consistent with an inflammatory response and pathology.

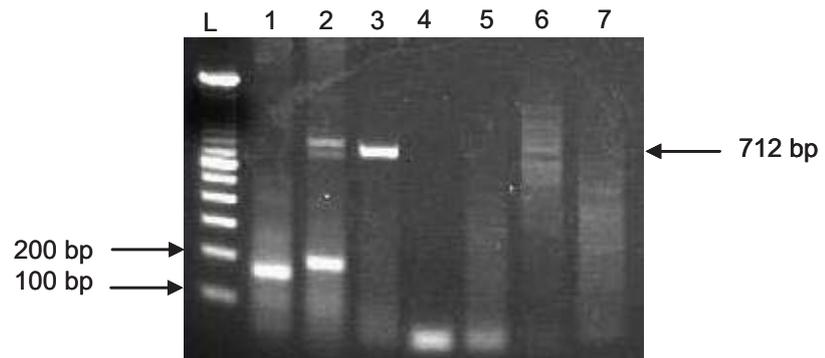


TNF- α gene expression in diseased articular tissues.

TNF- α (712 bp) gene expression was clearly evident in synovial fluid, AC and SCB of a 1 year old Romney ewe. Sample loadings were titrated at 5 ul and 2.5 ul. Lanes L: 100 bp DNA ladder, Lane 1: 5 ul synovial fluid, Lane 2: 2.5 ul synovial fluid, Lane 3: 5 ul AC, Lane 4: 2.5 ul AC, Lane 5: 5 ul SCB, Lane 6: 2.5 ul SCB, Lane 7: 2.5 ul liver (positive control tissue).

Appendix 7: Gene expression in a 3 year old Suffolk ram

Gene expression in liver and AC of a 3 year old Suffolk ram.



Gene expression in a 3 year old Suffolk ram

Lanes L: 100 bp DNA ladder, Lane 1 GAPDH (120 bp) liver, Lane 2: β -Actin (193 bp) liver, Lane 3: **TNF- α** (712 bp) AC, Lane 4: negative GAPDH liver, Lane 5: negative control β -Actin liver, Lane 6, 7 negative control **TNF- α** AC.

Appendix 8: Sheep training on the customized treadmill

Sheep were exercised in pairs within a customized cage on a generic equine treadmill situated adjacent to the sheep paddocks. Sheep were trained to freely enter the customized cage. The exercise program was conducted 3 days per week and structured to be osteo-inductive. It consisted of a brief walk and trot warm-up and then a short canter followed by gallop.



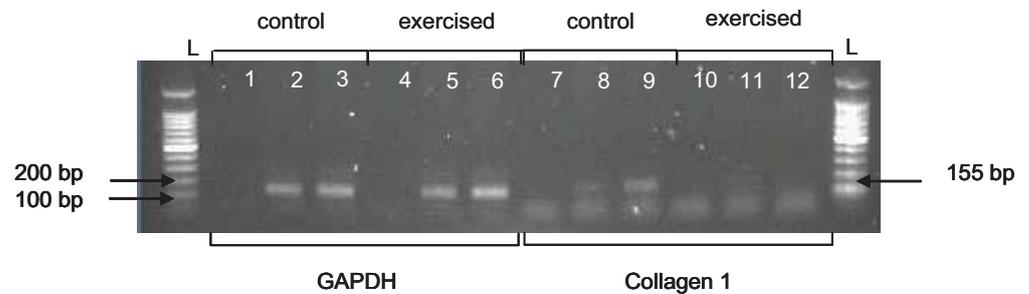
Sheep were trained to freely enter the customized cage on the generic equine treadmill.



Sheep during the short canter phase of the exercise program.

Appendix 9: Collagen type I gene expression in articular cartilage

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for collagen type I in AC of unexercised and exercised sheep. Collagen type I gene expression was 1.26718 – 1.3318 times greater in the AC of the unexercised animals compared to the exercised trained animals. The expression of collagen type I gene expression in adult AC is evidence of chondrocyte phenotypic change.

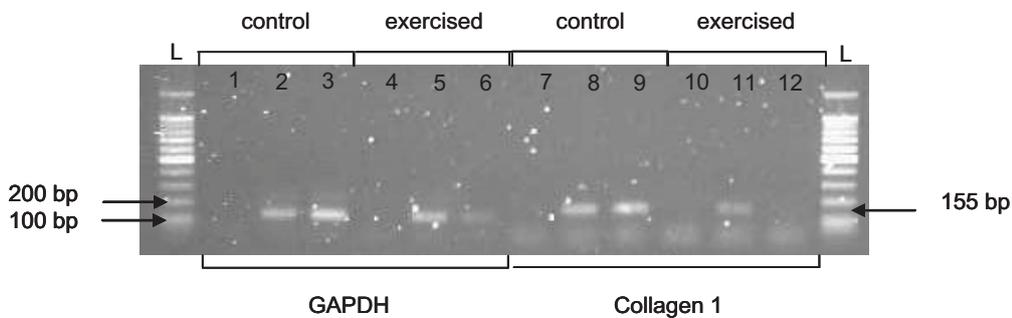


Collagen type I gene expression in articular cartilage of exercised and unexercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are collagen type I (155 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and collagen type I. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 10: Collagen type I gene expression in subchondral bone

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for collagen type I in SCB of unexercised and exercised sheep. Collagen type I gene expression was 1.2601 – 1.3195 times greater in the SCB of the unexercised animals compared to the exercised trained animals.

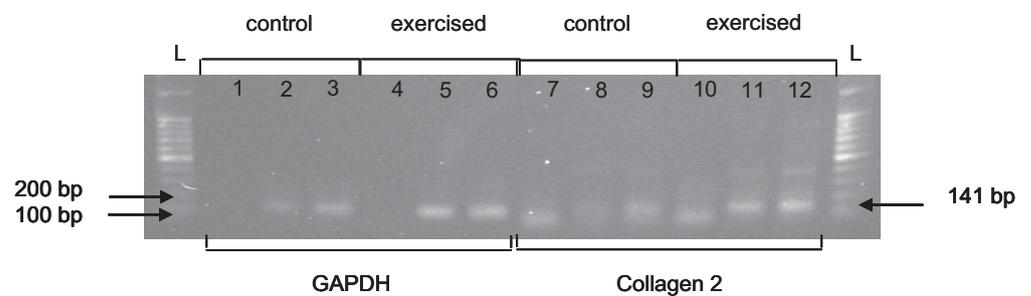


Collagen type I gene expression in subchondral bone of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-7 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are collagen type I (155 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and collagen type I. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 11: Collagen type II gene expression in articular cartilage

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for collagen type II in AC of unexercised and exercised sheep. Collagen type II gene expression was 1.1845 – 1.5650 times greater in the AC of the unexercised animals compared to the exercised trained animals.

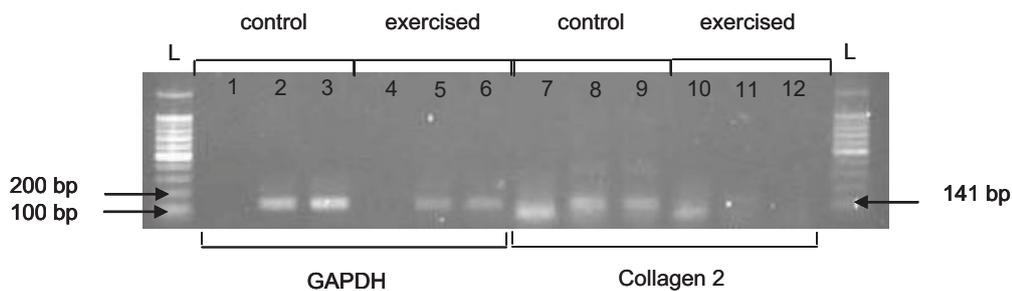


Collagen type II gene expression in articular cartilage of unexercised and exercised sheep.

Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are collagen type II (141 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and collagen type II. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 12: Collagen type II gene expression in subchondral bone

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for collagen type II in SCB of unexercised and exercised sheep. Collagen type II gene expression in the SCB of the unexercised animals was 1.1845 – 2.2981 times greater compared to no level of collagen type 2 gene expression in the SCB of the exercise trained animals. Expression of collagen type II in bone is evidence of a chondrocyte phenotypic change in response to exercise.

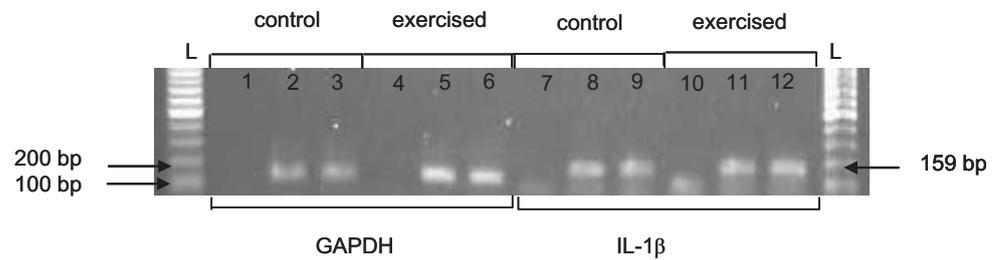


Collagen type II gene expression in subchondral bone of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are collagen type II for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and collagen type II. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 13: IL-1 beta gene expression in articular cartilage

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for IL-1 beta in AC of unexercised and exercised sheep. IL-1 β expression in the unexercised control animals was 1.1767 – 1.5388 times greater than that in the exercise trained animals.

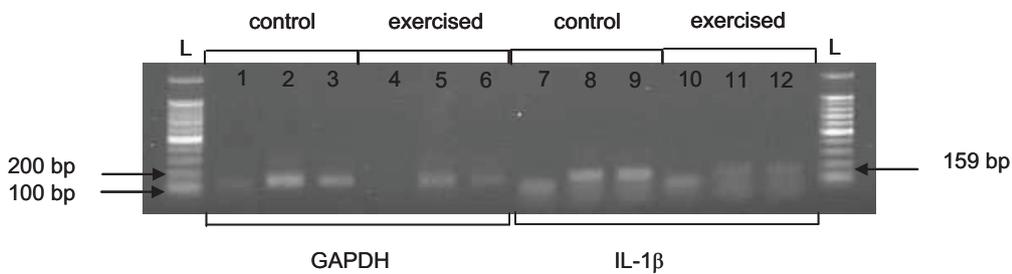


IL-1 β gene expression in articular cartilage of exercised and unexercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are IL-1 beta (159 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and IL-1 beta. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 14: IL-1 β gene expression in subchondral bone

GelDoc image of ethidium bromide stained electrophoretic gels of RT-PCR products for IL-1 beta in SCB of unexercised and exercised sheep. IL-1 beta expression is 1.0977 – 1.8155 times greater in the control animal compared to the level of expression observed in the exercise trained animal. Expression of IL-1 beta is indicative of an inflammatory response to exercise challenge.

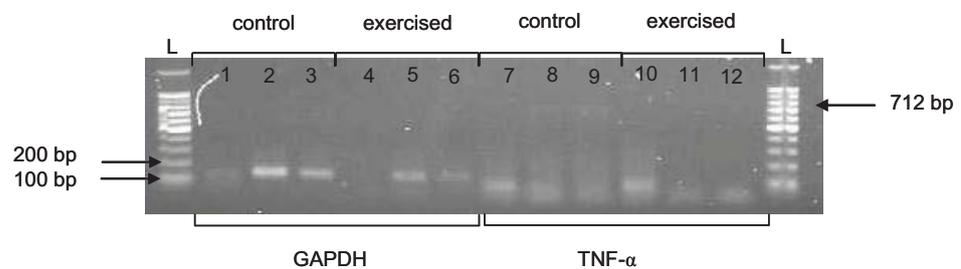


IL-1 beta gene expression in subchondral bone of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised sheep. Lanes 7-12 are IL-1 beta (159 bp) for unexercised and exercised sheep. Lanes 1, 4, 7 and 10 are negative controls for respective primers. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 15: **TNF- α gene expression in articular cartilage**

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for **TNF- α** in AC of unexercised and exercised sheep. A barely discernible level of gene expression may be seen in the untrained animal compared to no level of expression seen in the trained animal. However, no level of expression was evident in the SCB of any animals.

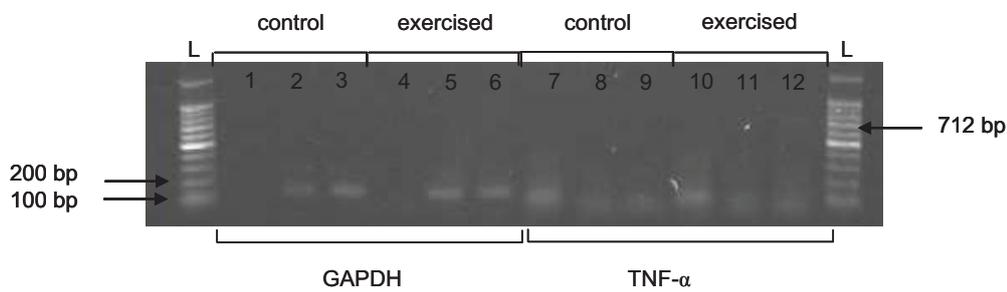


TNF- α gene expression in articular cartilage of exercised and unexercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised control and exercised animals. Lanes 7-12 are **TNF- α** (712 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and **TNF- α** . Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 16: TNF- α gene expression in subchondral bone

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for **TNF- α** in SCB of unexercised and exercised sheep. **TNF- α** expression was not evident in any of the SCB samples.

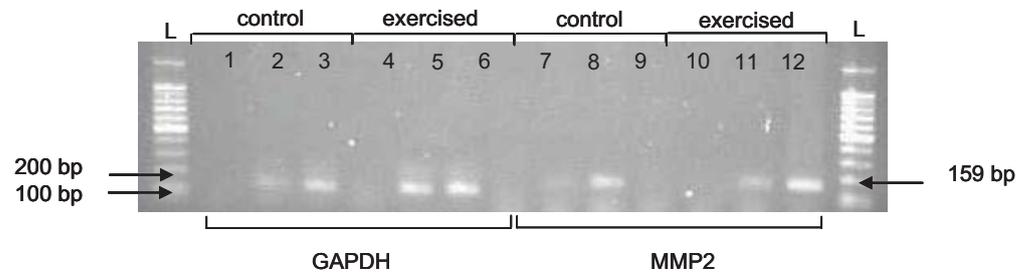


TNF- α gene expression in subchondral bone of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for control and exercised sheep. Lanes 7-12 are **TNF- α** (712 bp) for unexercised and exercised sheep. Lanes 1, 4, 7 and 10 are negative controls for respective primers. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 17: MMP2 gene expression in articular cartilage

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for MMP2 in AC of unexercised and exercised sheep. MMP2 gene expression was expressed in the AC of the unexercised animals at 1.155 – 1.765 times greater compared to low and no level of MMP2 gene expression in the AC of the exercise trained animals. The expression of MMP2 in AC is characteristic of active tissue degeneration.

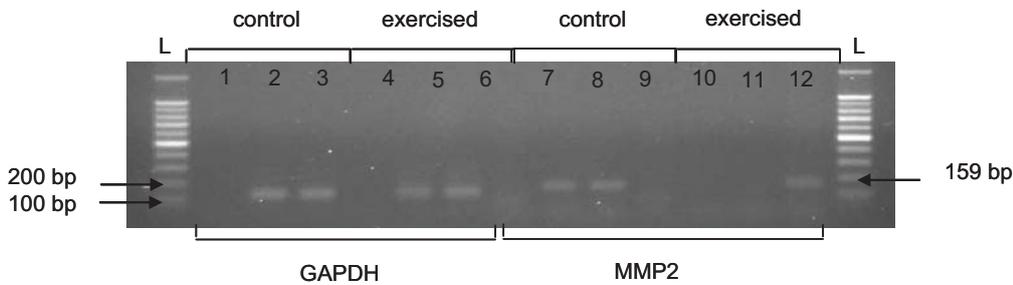


MMP2 gene expression in articular cartilage of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are MMP2 (159 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and MMP2. Lane 12 is positive control (uterine tissue, not run in duplicate) for MMP2. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

Appendix 18: MMP2 gene expression in subchondral bone

GelDoc image of ethidium bromide stained electrophoretic gel of RT-PCR products for MMP2 in SCB of unexercised and exercised sheep. MMP2 gene expression was expressed in the SCB of unexercised animals 1.1731 – 1.3846 times greater compared to no level of MMP2 gene expression in the SCB of the exercise trained animals. The expression of MMP2 in SCB is characteristic of active bone remodeling.



MMP2 gene expression in subchondral bone of unexercised and exercised sheep.

Lanes L are 100 bp DNA ladder. Lanes 1-6 are GAPDH (120 bp) for unexercised and exercised animals. Lanes 7-12 are MMP2 (159 bp) for unexercised and exercised animals. Lanes 1, 4, 7, 10 are negative controls for unexercised and exercised animals for primers GAPDH and MMP2. Lane 12 is positive control (uterine tissue, not run in duplicate) for MMP2. Lanes 2, 3, 5, 6, 8, 9, 11, 12 are positive samples for respective genes, run in duplicate.

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