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THE EFFECT OF EARLY EXERCISE ON THE ARTICULAR CARTILAGE AND SUBCHONDRAL BONE OF THE DISTAL THIRD METACARPAL/METATARSAL BONES OF YOUNG THOROUGHBRED HORSES.

A thesis presented in partial fulfilment of the requirements for the degree of Master of Veterinary Science at Massey University, Palmerston North New Zealand.

Susanne Dykgraaf

2003

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ABBREVIATIONS

BMD	Volumetric bone mineral density
CDET	Common digital extensor tendon
CLSM	Confocal laser scanning microscopy
DDFT	Deep digital flexor tendon
DJD	Degenerative joint disease
DXA	Dual x-ray absorptiometry
ECM	Extra-cellular matrix
HA	Hyaluronan
Il-1	Interleukin 1
Mc3	Third metacarpal bone
MCP	Metacarpophalangeal
MMP	Matrix metalloproteinases
Mt3	Third metatarsal bone
MTP	Metatarsophalangeal
NO	Nitric oxide
OA	Osteoarthritis
P_p	Proximal phalanx
PG	Proteoglycan
PI	Propidium iodide
pQCT	Peripheral quantitative computed tomography
PSB	Proximal sesamoid bones
ROI	Regions of interest
RA	Rheumatoid arthritis
SDFT	Superficial digital flexor tendon
SCT	Subchondral calcified tissues
SCB	Subchondral bone
TNF	Tumour necrosis factor

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ABSTRACT

The effect of early moderate exercise on articular cartilage and subchondral bone were investigated by comparing two groups of age and sex matched, pasture reared, 18 month old (± one month) Thoroughbred horses. The treatment group (n=6, 3 colts, 3 fillies) were exercised five days per week from 10 days of age on a purpose-built grass racetrack. The control group were managed identically but did not participate in an exercise programme.

Articular cartilage samples were taken from all horses, from the palmar and dorsal regions of the left and right distal third metacarpal bones (Mc3) and the palmar region of the left third metatarsal bone (Mt3). The sites were selected from regions that sustain high (palmar region) and low (dorsal region) load during exercise. The fresh articular cartilage samples were incubated with fluorescent stains (calcein-AM and propidium iodide) and examined under confocal laser scanning microscopy to assess chondrocyte viability. The number of viable and dead chondrocytes at each site was determined based on the fluorescent staining characteristics. The subchondral epiphyseal bone mineral density adjacent to the articular cartilage sample sites was measured using computed tomography data from regions of interest which were 2mm proximal to the interface of calcified cartilage and subchondral bone.

There was a 14% greater percentage of viable chondrocytes in the exercised horses (p=0.001), and a 34% greater percentage of viable chondrocytes at the control palmar regions compared to control dorsal regions (p=0.001). One exercised horse and five control horses had subtle macroscopic features consistent with osteoarthritis (OA) in the metacarpo(tarso)-phalangeal joints. Variation in chondrocyte viability was less in palmar and dorsal sites from exercised animals, and palmar sites from control animals. An association between percentage of dead chondrocytes and sclerosis of the subchondral bone (SCB) could not be identified. Lower chondrocyte viability occurred independently of SCB sclerosis. The sequence of initiating events leading to reduced articular cartilage viability appeared to be unassociated with SCB sclerosis at the sites of distal Mc3/Mt3 under the moderate exercise regimen imposed.

The effect of early exercise on the articular cartilage and SCB of young Thoroughbred horses has been further elucidated. Early exercise appeared to have beneficial effect on the viability of the articular cartilage sampled in this group of horses. The abnormalities detected may have been the earliest stages of idiopathic OA, but the relative and temporal involvements of articular cartilage and SCB remain undefined.

CHAPTER 1 INTRODUCTION

1.1 Background

Thoroughbred racing and breeding are multimillion dollar businesses providing livelihood and enjoyment to vast numbers of people. The industry in New Zealand alone has been estimated to contribute $256.1 million dollars per annum to GDP (Anon 1999).

During the 2001-02 New Zealand racing season 5,493 Thoroughbred horses competed in 2771 races for total prize money of $32,000,000 (New Zealand Thoroughbred Racing 2003). A similar number of horses are estimated to be involved in breeding, and equestrian sport, with an even greater number used for recreation. In Australasia considerable emphasis is placed on the production of 2 year old racehorses in response to the high stakes earnings available for a successful 2 year old (More 1999). Attitudes towards horse racing are strongly influenced by public perception, and opinion exists that training and racing of young horses with immature musculoskeletal systems is detrimental to their welfare (Lindner 1994). Scientific evidence on this opinion, and on the long term effects of training and racing young Thoroughbred horses, is limited (More 1999).

Premature loss of horses from racing and breeding can occur at any time from conception to maturity. The loss of racing animals, termed 'wastage', includes loss of potentially athletic animals, loss of training days, and all associated costs (Rossdale et al. 1985). Wastage of racehorses is most commonly attributed to a lack of athletic ability or the development of exercise associated injury (More 1999). Lameness has been repeatedly shown to be the most significant cause of wastage in young horses in training world-wide (Lindner and Offeney 1992; Jeffcott et al. 1982; Rossdale et al. 1985; Herzog and Lindner 1992; Bailey et al. 1997; Olivier et al. 1997; Perkins 1999). A study of 314 horses in training from the UK, found that over half of the horses (53%) experienced a period of lameness during one racing season, with 20% of the affected horses ultimately lost to racing (Jeffcott et al. 1982).

Joint related diseases are the most common cause of lameness in athletic horses (McIlwraith 1982; Todhunter 1996). Aetiologies of joint related lameness include developmental orthopaedic disease, acquired cartilage or bone damage (ranging from idiopathic primary osteoarthritis (OA) to osteochondral 'chip' fragments and catastrophic fracture), soft tissue trauma and infection. All of these conditions have the potential to lead to degenerative joint changes known as secondary OA (Todhunter 1996). True primary OA with fundamentally

defective articular cartilage has been found to occur in people with a type II collagen defect. However, primary OA similar to this has not yet been identified in horses (Caron 1999).

Osteoarthritis is a progressive and permanent deterioration of the articular cartilage, accompanied by subchondral bone (SCB) and soft tissue changes (McIlwraith and Vachon 1988). It may be thought of as a mechanically induced 'organ failure' with the synovial joint featured as the organ system (Radin and Rose 1986; Radin et al. 1991; Radin 1999). The question as to whether SCB changes precede or follow articular cartilage deterioration remains largely unanswered (Bailey and Mansell 1997; Martel-Pelletier 1998)

The role of SCB in initiating secondary OA has been studied for many years. Studies have shown SCB to spare the overlying articular cartilage from damage by attenuating axial mechanical loads (Radin and Paul 1971; Radin et al. 1970a; Radin et al. 1970b). It has been consistently reported that sclerosis of the SCB, manifested by trabecular (re)modelling, precedes articular cartilage damage (Radin et al. 1984; Radin and Rose 1986; Radin et al. 1991; Carlson et al. 1994; Dequeker et al. 1995; Burr 1998). Work by Radin et al on impact loading supports the theory that osteoarthritic changes are the result of repetitive impulsive loading leading to accumulated micro-trauma and healing micro-fracture. This scenario is characterised by articular cartilage thinning (Burr and Schaffler 1997) and degeneration that temporally follows stiffening of the SCB (Radin et al. 1973; Pugh et al. 1974; Dequeker et al. 1995). Radin and co-workers believe that as articular cartilage is compliant, it is unlikely to sustain micro-injury in the absence of SCB sclerosis (Radin 1999).

While acknowledging that changes in SCB are a factor in OA, a number of investigators believe the disease begins in the articular cartilage (Lee et al. 1974; Howell et al. 1976; Mankin and Brandt 1992). Primary biochemical changes induced by inflammatory mediators are thought to result in articular cartilage degeneration (Stephens et al. 1979; Mow et al. 1992; Wei et al. 2001). Primary articular cartilage damage has been found to occur without associated preceding SCB sclerosis (Donohue et al. 1983; Dedrick et al. 1993; Yamada et al. 2002).

Chondrocytes are involved in both the anabolic and catabolic responses of articular cartilage, and have been shown to respond to in-vitro loading (Lane Smith et al. 2000). The physiological and pathological effects of loading and exercise in vivo are difficult to determine and the exact relationship is uncertain. Articular cartilage composition and chondrocyte metabolism at a specific location within a joint are likely to be dependent on local variations in loading within the joint and the number of load cycles the joint sustains.

As chondrocytes are the only living element in articular cartilage, their viability is crucial for production and maintenance of the articular cartilage matrix. Biochemical alteration and mechanical disruption of the extra-cellular matrix collagen framework leads to loss of intrinsic tissue tension and leakage of proteoglycan (PG) aggregates. This results in an enhanced susceptibility to ongoing damage, which many see as the initial stage of osteochondral disease (Maroudas, 1976; Kempson 1979; Freeman 1980).

Unusually high forces are transmitted through the limbs of the Thoroughbred racehorse during galloping (Biewener 1993). As such, racehorses could be thought of as almost predestined for joint injury. During galloping the greatest vertical ground reaction forces are exerted by the leading forelimb on both the straight and turns (Back 2001). Following the initial impact shock and high frequency vibration of landing, the forces any joint sustains during the stance phase of the stride, are largely attenuated by the hoof capsule, vascular structures, joint tissues (articular cartilage and subchondral bone) and the soft tissues (muscular and tendinous structures) of the limb.

The forelimb is most commonly affected in musculoskeletal injury, contributing to approximately 90% of all racing injuries in Thoroughbreds. Eighty-five percent of the forelimb injuries are located between the carpus and the metacarpophalangeal (MCP) joint (Peloso et al. 1994). Exercise induced traumatic OA affects the high motion joints of the forelimb (McIlwraith and Vachon 1988; Pool 1996). The antebrachiocarpal, middle carpal and MCP joints are most commonly affected. Injuries are most often seen in animals that perform at high speeds or over distance. Factors affecting the incidence of musculoskeletal disease of racehorses include limb and hoof conformation (Chateau et al. 2001), lameness, hereditary factors and track surface (Peloso et al. 1994; Reiser 2000).

The MCP joint is a high motion, high load joint and has been reported to be involved in 14% of lameness cases involving Thoroughbred racehorses (Rossdale et al. 1985). During repetitive hyper-extension, the palmar condylar surfaces of distal Mc3 are under compressive and shear forces at the site of contact with the proximal sesamoid bones (Vilar 1995; Norrdin et al. 1998). The palmar aspect of the distal third of the metacarpal condyles is a consistent site of pathologic change due to exercise-induced overload osteoarthritis (O'Brien et al. 1981; Norrdin et al. 1998). The bone and soft tissue structures of the MCP joint are often involved in the development of OA and acute catastrophic racing injury (Pool and Meagher 1990).

Subchondral cancellous bone sclerosis has been shown to be a significant contributing factor to joint disease in racing Thoroughbred horses (Radin 1999). Sclerosis of the SCB in the palmar

aspect of Mc3 in response to training is hypothesised to result in a stiffness gradient, shear force concentration and micro-damage which may ultimately result in fatigue fracture or parasagittal fracture (Riggs et al. 1999a). The combination of rapid impulse loading and oscillatory movement occurring during the stance phase are believed to contribute to joint changes that can lead to OA and long bone fracture (Riggs 2002). This hypothesis is contrary to the notion that long bone fractures are 'spontaneous'. An aetiology similar to that of long bone fractures, involving accumulation of micro-damage in the SCB may initiate or speed the progression of OA (Riggs 2002). However the role of micro-damage in subchondral sclerosis, osteochondral fragmentation and OA in young horses in training has not been fully determined (Kawcak et al. 2001). Although debate continues as to the primary initiating events, there is agreement that both articular cartilage and SCB contribute to and are intimately involved in the manifestations of OA (Radin et al. 1982).

Exercise and loading can alter chondrocyte metabolism and/or viability. The effect of exercise on chondrocyte viability, articular cartilage composition and the development of pathologic change is not clear. As in human OA, the precise aetio-pathogenesis and the sequence of events involved in equine traumatic OA are not known. The sequence in the development of OA, along with the exact role of exercise, loading and micro-damage, remains to be studied in the horse (Norrdin et al. 1998). The success of all equine industries relies on minimising losses through successful management of the health and welfare of the horses. Preventing wastage of Thoroughbred racehorses due to lameness depends on the translation of scientific findings into practical training and management strategies aimed at reducing the incidence of joint-related injury and disease.

1.2 Literature Review

1.2.1 Synovial joint structure and function

The mobility and dexterity of vertebrates relies on functional synovial joints. The morphology of joints is closely related to the degree of motion which they facilitate. Three fundamental types of articulation are observed: synarthroses and fibrous joints which facilitate only a small degree of movement, and synovial or diarthrodial joints which provide an extensive range of near friction free movement. Synovial joints are formed between two articulating long bones covered by smooth articular cartilage (Johnston 1997). They facilitate angular movement during locomotion (Latshaw 1987) and are complex units of multiple tissues including articular cartilage, bone, synovium, ligament and joint capsule (Figure 1.1). Joints vary in size, shape and range of motion, but for all synovial joints normal function depends on the smooth, low friction, gliding, surface provided by articular cartilage. The joint is lubricated with synovial fluid to form a wear-resistant bearing surface with a low co-efficient of friction. Ligaments and capsule constrain the range and types of joint movements. The individual components of the synovial joint are described in more detail below.

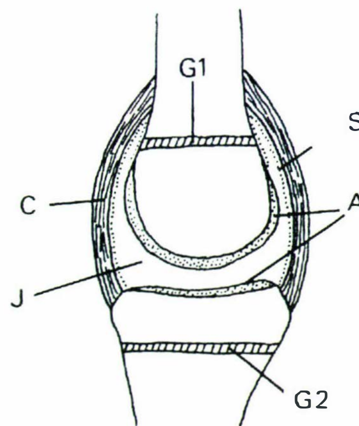


Figure 1.1: Diagram of a synovial joint.

The bones are covered with articular cartilage (A) and separated by a joint cavity (J). They are connected by a fibrous capsule (C), lined by a synovial membrane (S) that secretes synovial fluid. Note that the synovial membrane does not cover the articular surfaces of the joint. The site of attachment of the capsule determines whether or not a growth plate lies inside (G1) or outside (G2) the joint. ***From Biology of the Synovial Joint, Harwood Academic Publishers, The Netherlands (Benjamin 1999).***

1.2.2 Joint capsule and ligaments

The fibrous joint capsule and ligaments are soft tissue components that hold the ends of the long bones in place and influence the mechanics of the joint by permitting or limiting movement. The ligaments are local thickenings of the capsule designed to provide strength. Ligaments are found inside, outside and/or within the joint capsule. The capsule attaches immediately beyond the margin of the articular cartilage. The sites of attachment influence the range of motion of the joint. The capsule and ligament consist of type 1 collagen and elastin fibres with some fibroblasts and minor amounts of proteoglycan (Caron 1999). The structure of the capsule and ligaments changes from predominantly collagen to fibrocartilage and the fibrils ossify as they enter bone (Amiel et al. 1984). Blood vessels and nerves supply the capsule and ligaments (Johnston 1997).

1.2.3 Synovial membrane

The synovial membrane, or synovium, lines the non-articular surfaces of the joint. It facilitates joint movement and produces and absorbs synovial fluid. The synovium consists of 1-3 layers of cells (synoviocytes) supported by vascular connective tissue. Synoviocytes are classified into Type A macrophagic cells, Type B fibroblastic and secretory cells (secreting hyaluronan (HA) and other proteins) and Type C cells with intermediary functions (Caron 1999). Cross-over in functions has been identified and cell population appears to be dynamic depending on biological demands. Fibroblastic synoviocytes are also capable of secreting pro-inflammatory mediators which are involved in the aetiopathogenesis of OA (Caron 1999). The synovium frequently extends into the joint space as projections of villi or larger fatty folds. The villi and folds are compliant and can readily accommodate joint movements due to the fluid nature of the lipid within the synoviocytes at body temperature (Benjamin 1999).

1.2.4 Joint innervation and blood supply

The joint capsule, articular ligaments and synovium are richly innervated. As articular cartilage is aneural, innervation of the subchondral bone, periosteum and periarticular soft tissues is said to provide proprioceptive information (Caron 1999). The fibrous joint capsule is rich in pain-perceiving nocioceptors and position and load-perceiving mechanoreceptors (Johnston 1997).

The avascular nature of articular cartilage means that nutrients must migrate from blood vessels in the synovium into the synovial fluid and then diffuse into the articular cartilage matrix.

1.2.5 Synovial fluid and lubrication

1.2.5.1 Synovial fluid

Synovial fluid is a clear, straw coloured highly viscous liquid secreted into joint cavities by the synovium. It is a dialysate of blood plasma containing long chain protein molecules and HA. Synovial fluid lubricates the joint, provides the nutritional needs of the articular cartilage and acts as a medium for osmosis of metabolic by products between the articular cartilage and the adjacent synovial membrane.

1.2.5.2 Lubrication

Articular cartilage exhibits properties unattainable by man-made materials (Graindorge and Stachowiak 2000). It provides a relatively wear-resistant surface with low frictional properties for joint motion even under high loads. Friction is the resistance to movement of two surfaces sliding over one another expressed as a co-efficient of friction (Smeathers 1992). In tests conducted on synovial joints a friction co-efficient as low as $\mu = 0.003$ has been reported (Swanson 1979).

The surface roughness of articular cartilage changes with age and degeneration, the significance of surface changes in lubrication regimens is uncertain as the compliance of the articular cartilage allows alteration of the shape of the articular surface under load (Smeathers 1992).

Lubrication minimises frictional resistance between bearing surfaces by keeping them separated (Radin and Paul 1972) and keeps wear of articular cartilage to a minimum (Mow and Soslowsky 1991). A number of lubrication theories have been proposed to explain the low wear and frictional characteristics of articular cartilage (Radin and Paul 1972; Mow and Soslowsky 1991; Clark 1999). A combination of lubrication modes is most likely to occur within a synovial joint at one time. Two fundamental theories of lubrication are boundary lubrication and fluid-film lubrication (Mow and Soslowsky 1991).

a) Boundary lubrication

Boundary lubrication (Figure 1.2) acts by chemical repulsion of two surfaces due to a monolayer of lubricating glycoprotein preventing adhesion and abrasion of the opposing surfaces (Johnston 1997). Synovial fluid is absorbed onto the surface of articular cartilage protecting the surfaces from wear. This form of lubrication is relatively insensitive to load, viscosity of the lubricant, and stiffness of the contacting material. It is important for static loading conditions (Smeathers 1992).

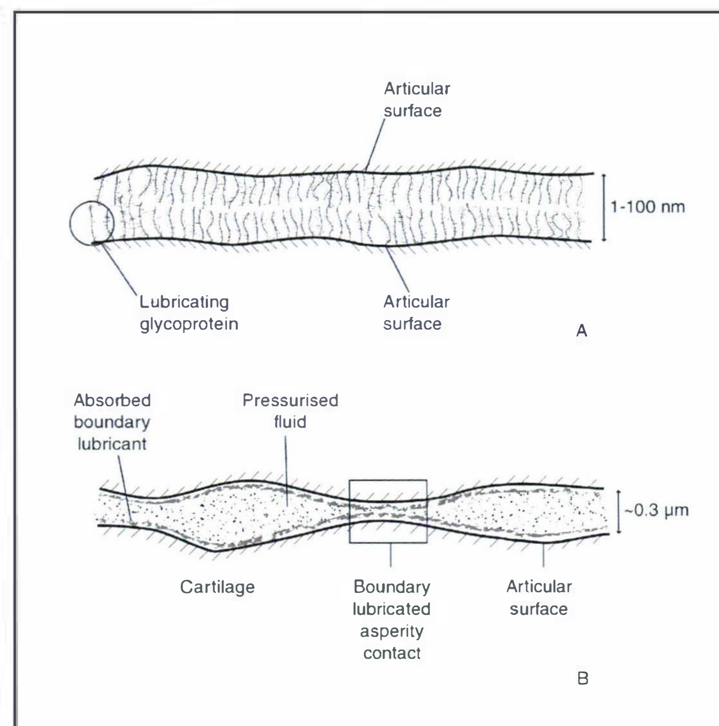


Figure 1.2: Boundary lubrication of articular cartilage.

A: In this mode, the load is carried by a monolayer of lubricating glycoprotein which is absorbed onto the articular surfaces. B: Boundary lubrication occurs where the thickness of the fluid is of the same order as the roughness of the bearing surface. Fluid film lubrication takes place in areas with more widely separated surfaces. *From Basic Orthopaedic Biomechanics, Raven Press Ltd, New York (Mow and Soslowsky 1991).*

b) Fluid lubrication

Under high loads boundary lubrication can not function effectively (Radin and Paul 1972). Frictional forces can be reduced with fluid film lubrication due to fluids confined between two sliding surfaces. This fluid film lubrication occurs when fluid pressure maintains separation of the articular surfaces, and occurs as hydrodynamic and squeeze film lubrication (Figure 1.3).

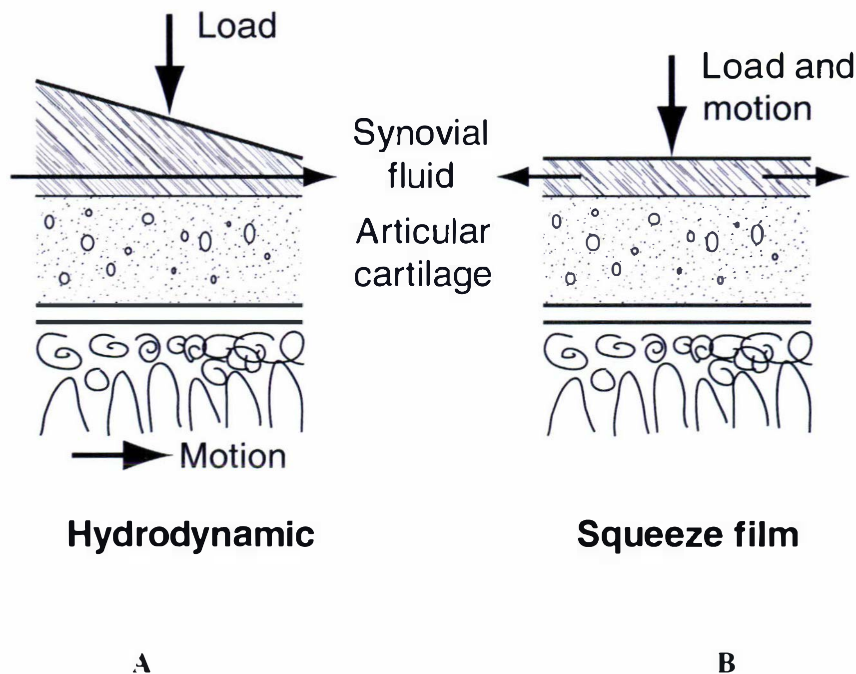
- Hydrodynamic

The separating pressure is developed by surfaces that are moving transversely over each other at high speed. A wedge of fluid is formed creating hydrodynamic pressure and generating lift, forcing the two surfaces apart. Hydrodynamic lubrication is especially effective when the surfaces are deformable, providing more suitable gap geometry, when it is then termed elasto-hydrodynamic lubrication, the lubrication mode typical of continuous rapid movements of synovial joints.

The mechanism can not operate alone as synovial joints are not constantly operating at high speed (Mow and Soslowsky 1991). The long molecules in synovial fluid align parallel to the direction of flow and the viscosity of the fluid decreases as the shear rate increases (Smeathers 1992).

- Squeeze film

Squeeze film lubrication occurs under more static conditions (Mow and Soslowsky 1991). Pressure is built up because the fluid takes time to be forced out of the gap between the closing surfaces, such as occurs during short term static loading and during changes in direction of movement when the joint is loaded. Eventually, under prolonged static loading, this gap closes and boundary layer lubrication takes over.



A B

Figure 1.3: Hydrodynamic and squeeze film fluid lubrication.

A: Schematic representation of hydrodynamic lubrication. Viscous fluid is slowly forced out of a convergent channel causing a pressure field to be generated in the lubricant. Fluid viscosity, gap geometry, and relative sliding speed determine the load carrying-capacity. B: As the bearing surfaces are squeezed together, the viscous fluid is forced from the gap in the transverse direction. This squeeze action generates hydrodynamic pressure for load support. The load carrying capacity depends on the size of the surfaces, velocity of approach, and fluid viscosity. *Modified from Basic Orthopaedic Biomechanics, Raven Press Ltd, New York (Mow and Soslowsky 1991).*

1.2.6 Articular cartilage

The cartilage covering the ends of long bones is that which has escaped endochondral ossification (Benjamin 1999). Articular cartilage differs in thickness, cell density, matrix composition and mechanical properties among species (Athanasiou et al. 1991), between and within individuals (Malinin and Ouellette 2000), and between sites in the same joint. However, all articular cartilage consists of the same basic components, and has a similar structure and function. Healthy articular cartilage is an avascular, aneural and alymphatic tissue whose composition, structure and function are closely related.

1.2.6.1 Growth and development of articular cartilage

Mesenchymal cells aggregate and condense to form a blastema as a prelude to bone formation. A three layered interzone forms within the blastema between the ends of the bone models and is central to the formation of the joint. Two chondrogenic layers continuous with the perichondrium form cartilage apposed to their respective bone ends. A membranous synovial mesenchyme encases the interzone and forms the articular capsule. Condensations of the blastemal tissue within the capsule form ligaments. The synovial mesenchyme in the centre of the interzone undergoes cavitation to form a joint cavity which is lined by synovial membrane (Benjamin 1999).

Formation of the major joints has occurred by the end of the embryonic period. Vascularisation and innervation of the articular structures occurs during the foetal period. Muscular activity in utero is necessary for the normal development and maintenance of synovial joints (Latshaw 1987). The cellular and molecular events that control the development of joints and articular cartilage are not well known (Benjamin 1999).

During formation and growth of articular cartilage the cell density is high and the chondrocytes proliferate rapidly to synthesise large volumes of matrix. When the chondrocytes assume spherical shape, begin to synthesise collagens, proteoglycans, non-collagenous proteins, and become separated by matrix, the tissue becomes recognisable as articular cartilage by light microscopy. It is currently believed that after completion of skeletal growth the majority of normal chondrocytes continue to synthesise matrix macromolecules but never divide (Shum and Nuckolls 2002). Continued synthetic and degradative activity suggests that maintenance of articular cartilage requires ongoing internal remodelling of the matrix framework (Mort and Billington 2001).

1.2.6.2 Structural arrangement of articular cartilage

a) Macroscopic appearance

On gross examination articular cartilage is smooth, glossy and bluish-white, with a slick firm surface that resists deformation (Nilsson and Olsson 1973). The thickness varies both within a joint and between joints of the same individual. Variations in articular cartilage thickness across the joint improve the congruity of the apposing surfaces.

b) Microscopic appearance

On light microscopic examination, articular cartilage consists of an elaborate structure of cells and extra-cellular matrix (Buckwalter et al. 1987). A variety of complex interactions between the chondrocytes and the matrix actively maintain the tissue (Fassbender 1987).

In normal articular cartilage, chondrocytes are distributed in an ordered fashion throughout the extra-cellular matrix. The general organisation of the chondrocytes and collagen fibres in the matrix leads to classification of the articular cartilage into four zones. The zones from the articular surface to subchondral bone are, 1) superficial or tangential zone (10-20% of linear distance), 2) middle or intermediate zone (40-60%), 3) deep or radial zone (20-50%) (Mow and Wang 1999), and 4) calcified cartilage zone (Stockwell 1971; Aydelotte and Kuettner 1988) (Figure 1.4).

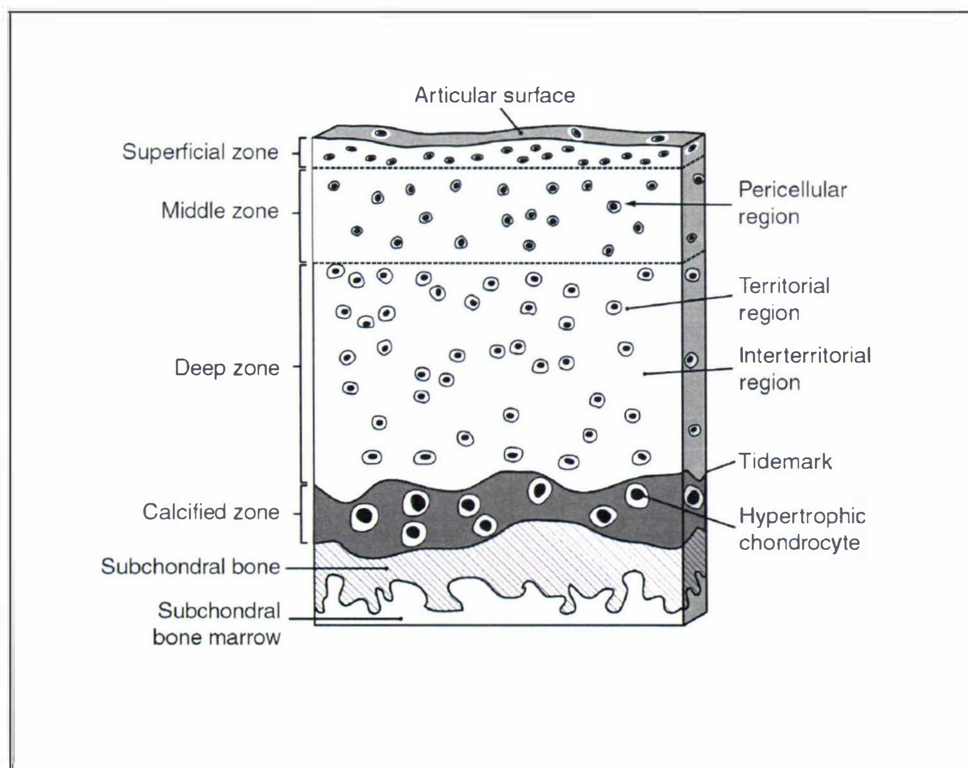


Figure 1.4: Regional organisation of mammalian articular cartilage.

Adapted from Equine Surgery 2nd Edition, W.B. Saunders Company, Philadelphia (Caron 1999)

The transition between zones is often not well defined (Stockwell 1979; Schenk 1986). However, identification of the individual zones is useful for comparative purposes (Bush and Hall 2001b). The superficial layer contains small flattened and elongated cells with their axes parallel to the surface of the articular cartilage. The intermediate layer has chondrocytes arranged in a more random fashion before becoming oriented in columns perpendicular to the articular surface in the deep zone.

The articular cartilage is attached to the SCB by the transitional zone of calcified cartilage, which consists of sparse chondrocytes within lacunae surrounded by collagen fibrils in a mineralised matrix (Martinelli et al. 2002). The junction between the deep and the calcified cartilage zones is termed the tidemark or tideline (Oegema et al. 1997). The tidemark is histologically demonstrated by a thin gently undulating basophilic line interlocking the two adjacent layers. The tidemark demarcates the non-calcified from the calcified cartilage (Redler et al. 1975; Bullough and Jagannath 1983), providing a transition between two dissimilar regions of cartilage (Bullough and Jagannath 1983; Radin and Rose 1986). Multiple tidemarks may be present in normal joints of all species and are not specifically indicative of OA (Oegema et al. 1997).

As articular cartilage and the adjacent SCB are a functional unit, the tidemark has significant biomechanical functions as it remodels in response to loading or micro-injury (Imhof et al. 1999). Remodelling of the calcified cartilage has previously been shown to cause thinning of the non-calcified articular cartilage and to alter the state of stress within the tissue (Bullough and Jagannath 1983). However equine middle carpal joint calcified cartilage thickness has been shown to increase in response to exercise, while non-calcified articular cartilage thickness remains unchanged (Murray et al. 1999).

1.2.6.3 Chondrocytes

The chondrocyte is the lone cell type in normal articular cartilage. It is highly specialised and terminally differentiated to produce extra-cellular matrix of predominantly type II collagen, PG and water (Buckwalter et al. 1989; Maroudas 1979). Chondrocytes contribute less than 10% to the volume of the articular cartilage by weight or volume (Mow and Wang 1999), but as the only living element these cells are of great importance. As chondrocytes have a low metabolic rate, articular cartilage is tolerant of near anaerobic conditions and individual chondrocytes may remain alive for days after clinical death before succumbing to autolysis (Gardner 1990).

Nutrients must pass from synovial capillaries through the synovial membrane, synovial fluid and articular cartilage matrix to reach the chondrocytes. The multiple barriers limit the types and concentrations of nutrients available to the cells. The matrix restricts not only the size of the nutrients, but also their charge and molecular configuration.

It has been shown that although the chondrocyte is specialised to create and survive in a near anaerobic environment, it is as complex and active as other more differentiated cells (Stockwell 1990). Chondrocytes within the articular cartilage differ in size, shape and possibly metabolic activity (Buckwalter and Hunziker 1999).

Chondrocytes do not form cell-to-cell contacts. Matrix covers and binds to the cell membranes. Organelles that facilitate matrix synthesis include endoplasmic reticulum, golgi apparatus, intracytoplasmic filaments, lipid, glycogen, and secretory vesicles. Histochemical differences have been found in the macromolecular composition of the matrix, leading to classification of the matrix into pericellular, territorial and interterritorial regions.

Chondrocyte synthetic rates are altered by intrinsic (e.g. matrix solute concentration) and extrinsic (cyclic mechanical loading) factors (Palmoski and Brandt 1984; Schneiderman et al. 1986). The poor regenerative capacity (Riddle 1970) and low metabolic rate of articular cartilage has fostered the notion that the tissue is relatively inert (Ryu et al. 1984) and chondrocytes effete. However chondrocytes react to their environment and are osmotically and electrochemically sensitive (Mow and Wang 1999).

1.2.6.4 Matrix composition

The extra cellular matrix (ECM) consists of water, proteoglycan (PG) and collagen (Figure 1.5). The framework of collagen and PG provides tissue stability, maintains tissue fluid within the matrix, and provides the mechanical properties of compressive stiffness and resilience to articular cartilage (Buckwalter and Hunziker 1999). The biomechanical properties of articular cartilage depend on the integrity of the cartilage matrix. The biochemical composition of equine articular cartilage is similar to that of other species (Vachon et al. 1990).

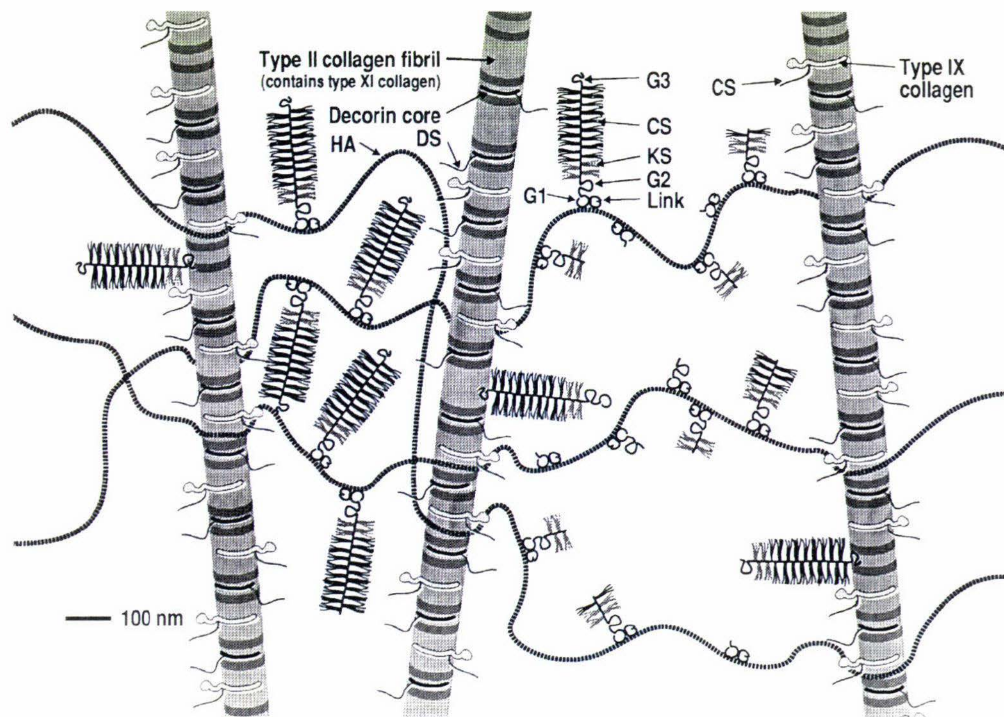


Figure 1.5: Organisation of the major extra-cellular matrix components in articular cartilage.

Organization of type II collagen fibrils, containing type IX and type XI collagens and the proteoglycan aggrecan, which binds HA in the extra-cellular matrix of articular cartilage. Type IX collagen is covalently bound to and has a periodic distribution on type II collagen. Decorin also binds to type II collagen through its core protein, and its single dermatan sulphate (DS) chain is shown. Intact aggrecan molecules (mainly on the left side of figure) contain a G_3 globular domain at the C terminus. They are attached to HA via a globular G_1 N-terminal domain. This binding is stabilized by link protein. The G_2 globular domain (also G3) has no known function. Keratan sulphate (KS) and chondroitin sulphate (CS) are bound to core protein as shown. Degradation products of aggrecan are shown (right side of figure) and remain bound to HA via the functional G_1 domain. The nature of the direct association of aggrecan with collagen fibres remains to be established. ***Adapted from Arthritis and Allied Conditions: A textbook of Rheumatology, Lea & Febiger, Philadelphia (Poole 1993).***

a) Water

Water is the major component of the extra-cellular matrix (Ratcliffe 1996), being up to 80% of the wet weight. Some of the water can move freely in and out of the articular cartilage. Its volume, concentration and behaviour depends on the interaction with the large hydrophilic, negatively charged, aggregated PGs. The cartilage fluid contains gases, small proteins, metabolites and a high concentration of cations (<1% by wet weight) to balance the negatively charged PG (Mow and Wang 1999). The large PGs help maintain the fluid and electrolyte concentrations within the matrix.

b) Proteoglycans (PGs)

Proteoglycans consist of one or more glycosaminoglycan (GAG) chains covalently attached to a protein core (Todhunter 1996), and contribute approximately one third of dry weight of adult articular cartilage. A PG monomer is composed of a core protein to which one or more types of GAG chains are attached (Johnston 1997). Proteoglycans are principally involved with resisting compression through osmotic attraction of water and creation of swelling pressure associated with their fixed charge density (Mow and Wang 1999). Under physiological conditions the stiff collagen fibres prevent PGs from taking up water maximally.

Articular cartilage PGs include the large aggregating PGs, the aggrecans, non-aggregating large PGs. A number of smaller, less abundant PGs (decorin, fibromodulin and biglycan) are also synthesised by chondrocytes (Miller and Matukas 1969; Rosenberg et al. 1985; Aydelotte et al. 1988; Sommarin et al. 1989; Oldberg et al. 1989). Aggrecan is the largest and most abundant PG in the extra-cellular matrix (Roughley 2001). The GAG chains consist of two regions, a linkage region and a repeating disaccharide. Aggregating proteoglycans contain chondroitin sulphate and in most species keratin sulphate (Figure 1.6).

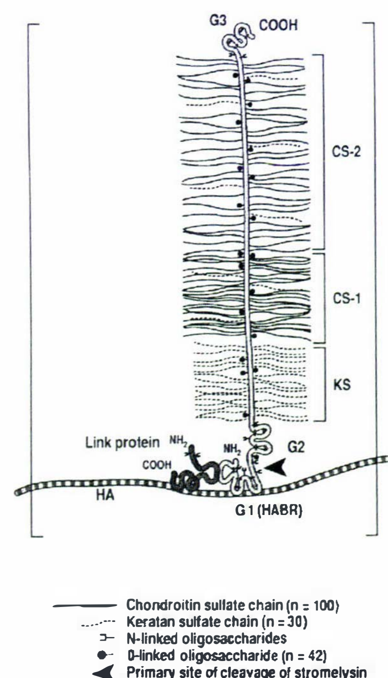


Figure 1.6: Diagrammatic representation of the large proteoglycan aggrecan and link protein, which bind to each other and to hyaluronan (HA).

A primary proteinase cleavage site between G_1 and G_2 is shown by the arrow. CS = chondroitin sulphate; KS = keratan sulphate; HABR = HA-binding region. ***From Arthritis and Allied Conditions: A Textbook of Rheumatology, Lea & Febiger, Philadelphia (Poole 1993).***

The non-aggregating PGs are divided into two groups, chondroitin sulphate-dermatan sulphate PGs and keratin sulphate PGs. The leucine-rich small PGs are decorin, biglycan and fibromodulin. The understanding of the exact function of the small non-aggregating PGs is limited (Todhunter 1996). Small non-collagenous, non-PG link proteins also make up a fraction of the articular cartilage.

Proteoglycans found in equine articular cartilage, with the primary protein core in brackets, are: chondroitin sulphate (aggrecan), dermatan sulphate (decorin and biglycan), keratan sulphate (aggrecan and fibromodulin), hyaluronan (none) and heparin sulphate (none) (Todhunter 1996). Hyaluronan is unique in that it is not synthesised attached to a core protein. Most hyaluronan is involved in large aggregates, and is the only glycosaminoglycan that is not sulphated (Todhunter 1996). The aggrecans interact with hyaluronan through non-covalent bonds to yield a hydrated array of negatively charged macromolecules to resist compression (Hardingham and Muir 1972; Maroudas 1979).

Proteoglycan turnover is relatively rapid. The distribution of PG within the matrix is related to biomechanical requirements and varies markedly from joint to joint, geographically within a single joint, and with aging (Brama et al. 1999b; Brama et al. 2000; Brama et al. 2000b).

c) Collagen

Superficially, collagen fibrils are packed close together and oriented parallel to the articular surface in a protective manner. The intermediate layer consists of randomly distributed collagen bundles whereas deeper collagen fibrils are arranged radially to anchor the articular cartilage to the underlying bone plate. In general collagen fibre thickness increases by a factor of four from the superficial zone to the deepest layer (Bullough 1992). The deep zone collagen fibrils are anchored within the calcified cartilage (Imhof et al. 1999). The nature of the lattice constrains GAGs and water, contributing to the compressive stiffness of articular cartilage.

Collagen contributes approximately two-thirds of the dry weight of adult articular cartilage (Eyre 2002) and interacts with other matrix components to provide structural support (Askew 1978; Caron 1999). Types of collagen synthesised include the major articular cartilage protein, type II collagen, and a number of minor forms such as type I, type V, type IX and type XI (Reese and Mayne 1981; Eyre et al. 1987). The major collagen, type II collagen, interacts with aggrecans and water through covalent, ionic and hydrogen bonding to form a stabilised matrix. Type II collagen is organised into triple helix fibrils and forms a fibrous network of these cross-linked fibrils that is stabilised in part by small proteoglycans and the minor collagens (Types VI,

IX, XI, XII, XIV). Fibronectin, an adhesive glycoprotein, mediates cell-matrix interactions and collagen network organisation. There is little capacity for the overall collagen network to be reproduced if damaged (Eyre 2002).

1.2.6.5 Extra-cellular matrix metabolism and turnover

Maintenance of the articular cartilage matrix involves a balance between chondrocyte-mediated synthesis and degradation (Caron 1999). The long-term matrix stability depends on the metabolic state of the chondrocytes, as a change may alter the type of PG and collagen expressed and influence the release of matrix degrading enzymes (Benya et al. 1988). The collagen network is long-lived, whereas the PGs are subject to constant turnover with a half life suggested to be between 7-200 days dependent on joint and measurement method (Fassbender 1987). Turnover of the matrix macromolecules allows tissue to be modified in response to joint use and mechanical loading as occurs during weight-bearing and exercise (Sah et al. 1989).

Using a fluid-induced shear stress model for stimulation of chondrocytes in vitro, it was shown that articular chondrocytes exhibit a dose and time-dependent response to shear stress that results in release of soluble mediators and extra-cellular matrix molecules. This suggests that the chondrocyte response to mechanical stimulation contributes to the maintenance of articular cartilage homeostasis in vivo (Lane Smith et al. 2000).

Chondrocytes detect and respond to changes in the matrix composition through interactions termed 'dynamic reprocicity' (Scully et al. 2001). Chondrocytes are implicated as the mediators of damage to collagen molecules within the matrix they have produced (Goldring 2000). Chondrocytes both synthesise and initiate activation of matrix metalloproteinases (MMPs) (Hollander 1997). An imbalance between MMPs and their tissue inhibitors (TIMPs) may contribute to articular cartilage degradation (Billinghurst et al. 1997; Mort and Billington 2001). MMPs can be divided into four main classes, collagenases, gelatinases, stromelysins and membrane-type (Clegg and Carter 1999), some of which can be synthesised by chondrocytes. There is some evidence that fragments of fibronectin can stimulate chondrocyte-mediated articular cartilage resorption via cell surface receptor activation (Homandberg et al. 1992). Fibronectin is produced in increased amounts in OA cartilage (von der Mark et al. 1992; Murray et al. 2000).

Activation of chondrocyte catabolism by matrix degradation products may play an important part in establishing a positive feedback cycle of proteolysis. Cellular responses produce

multiple proteases and specific inhibitors that maintain equilibrium between degradation and synthesis. Human, rabbit and equine chondrocytes have been shown to respond to mechanical or chemical stimuli by producing inflammatory mediators (May et al. 1992).

Cytokines produced by chondrocytes influence matrix stability and include matrix degrading enzymes interleukin 1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor (TNF) and also growth factors such as insulin-like growth factor I (IlGF-1) and transforming growth factor-beta (TGFβ) and growth factor binding proteins (Hardingham 1979; Tyler et al. 1992; Woods et al. 1994; Trippel 1995; Olney et al. 1996). There is enhanced expression of IL-1 and tumour necrosis factor (TNF) by OA chondrocytes and synovium (Arner and Tortorella 1995; Melchiorri et al. 1998). Il-1 and TNF are potent activators of articular cartilage degradation in vitro (Poole 1995; Poole 1997). Nitric oxide (NO) can induce apoptosis in chondrocytes (Blanco et al. 1995) but may also play a protective role in reducing protease activity (Stefanovic-Racic et al. 1996; Bird et al. 2000a). A deficiency in tissue inhibitors of MMPs favours excessive matrix degradation in OA (Dean et al. 1989).

In animal models two types of repair reactions occur, depending on the thickness of the defect. Full thickness osteochondral defects heal by production of granulation tissue from the subchondral bone, which undergoes metaplasia into fibrocartilage (Henrotin and Reginster 1999). The repair tissue is characterised by chondrocyte clustering (mitosis and or migration), changes in matrix composition, and articular cartilage hypertrophy. The quality of the reparative tissue is inferior to original articular cartilage, and degenerates with time and use, in two phases, the biosynthetic and the degradative phases. Chondrocytes initially attempt to repair damage, however eventually anabolic activity is unable to keep pace with catabolic activity, resulting in net loss of proteoglycan (Figure 1.7) (Mankin et al. 1971; Sandell and Aigner 2001).

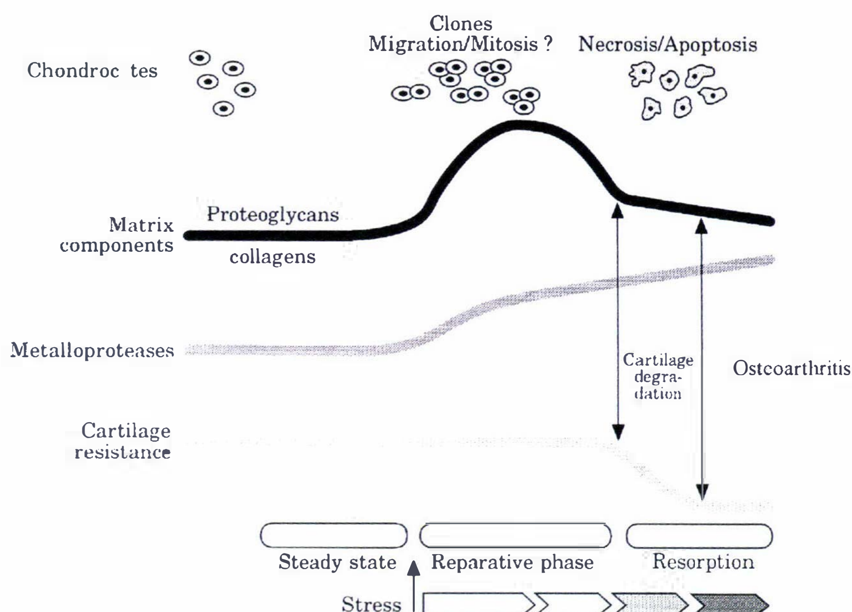


Figure 1.7: The reparative attempt of chondral defects in which injury is limited to articular cartilage.

From Osteoarthritis and Cartilage (Henrotin and Reginster 1999).

Zonal variation in the anabolic response of chondrocytes appears to occur, with cells of the upper zone down-regulating their expression of matrix components, particularly aggrecan. The suppression of anabolic activity of chondrocytes in the upper zone contributes to the metabolic imbalance of OA cartilage (Aigner et al. 1997). The chondrocytes progressively decompensate, start secreting more catabolic and proteolytic cytokines, and finally die most likely by apoptosis (Henrotin and Reginster 1999). The deeper zone chondrocytes are still activated and producing matrix molecules (Aigner and Dudhia 1997). Even in severe OA (Mankin scores greater than 8), chondrocytes of the middle and deep layers keep their anabolic capacity (Sandell and Aigner 2001).

Under different surface loads each articular cartilage zone experiences different strains, with the superficial layer experiencing greater strains (Wong et al. 1997). It is thought that during degenerative disease the chondrocytes at the articular surface either die or are sloughed off during fibrillation. During endochondral ossification, cartilage grows from the surface region, indicating the presence of articular cartilage progenitor cells in that region. It is possible that the cells which possess developmental capability to regenerate articular cartilage are lost during the disease process, or that once articular cartilage matures the cells responsible for its growth lose the capacity for further tissue regeneration (Archer and Francis-West 1999).

1.2.7 Subchondral bone

Bone is an abundant matrix of Type 1 collagen fibrils and mineral (mostly calcium and phosphate). Bone underlying articular cartilage is biochemically and histologically similar to bone in other locations, but its organisation is specific to its subarticular site (Caron 1999). The adult articular and calcified cartilages are supported by a thin layer of cortical bone, termed the subchondral bone-plate. The articular cartilage interdigitates with the subchondral plate forming an irregular junction. Underlying the subchondral plate is cancellous bone consisting of trabecular pillars or struts separated by marrow spaces (Noble and Alexander 1985) which contain vascular channels for nutrient supply to the subchondral bone plate and deeper layers of articular cartilage inaccessible to synovial fluid (Kawcak et al. 2001).

The subarticular tissues consisting of subchondral cortical bone plate and adjacent epiphyseal trabecular bone, can be collectively referred to as the subchondral bone (SCB). The SCB provides support for the overlying non-calcified articular cartilage to maintain joint surface contour and attenuate mechanical force applied through joints (Radin and Paul 1970; Radin and Paul 1971; Pugh et al. 1973b). SCB adapts readily to mechanical forces and exercise (Simkin et al. 1991; Oettmeier et al. 1992).

Articular cartilage fails to significantly alter peak dynamic forces across whole joints and SCB absorbs most of the impact under loading conditions (Radin and Paul 1970). The remaining force attenuation is taken up by cortical bone, joint capsule and synovium. Although the peri-articular soft tissues (ligaments, capsule and synovium) have an effect, the major structural component which attenuates frictional and longitudinally applied force is the supporting bone (Radin and Paul 1971). The transfer of load from the SCB to cortical bone results in large shear stresses at the SCB-cartilage interface (Imhof et al. 1999). The variation in density and thickness of SCB across the joint surface leads to a variation in mechanical properties and cushioning ability at each site (Kawcak et al. 2001).

In 1892 Julius Wolff suggested that changes in the loading of bones caused mathematically related changes in their structure which has come to be known as Wolff's Law (Buckwalter and Grodzinsky 1999). Bone responds to changes in the mechanical strain environment by modelling or remodelling. Remodelling involves the removal and replacement of bone in response to micro-damage (Kawcak et al. 1998). It occurs in a sequence of activation-resorption-formation (Burr et al. 1989) involving replacement of 'packets' (Kawcak et al. 2001) of damaged bone (Frost 1990). Modelling is the changing of bone shape to withstand the loads placed on it by activation of the bone followed by independent resorption or formation of bone

(Burr et al. 1989). Modelling can occur at an organ level (macro-modelling) or trabecular level (micro-modelling) (Kawcak et al. 2001). In an optimum strain environment a balance of formation and resorption is maintained (Kawcak et al. 2001). Osteocyte viability is thought to affect the response of bone to strain. Osteocytes detect changes in the local strain environment and may stimulate bone (re)modelling accordingly. Loss of osteocyte viability may lead to osteoclastic activity (Noble et al. 1997). Trabecular modelling and remodelling results in less compliant SCB and transfers excessive mechanical stress to the overlying articular cartilage (Simon et al. 1972; Radin et al. 1973; Burr et al. 1985). During elastic deformation of cancellous bone bending or buckling of trabeculae may occur (Pugh et al. 1973c). This may be a physiological shock absorption mechanism preceding physiological or pathological trabecular micro-fracture.

Fatigue micro-damage has been shown to be a significant factor in the initiation of bone remodelling (Burr et al. 1985). If micro-damage formation exceeds repair then gross damage may occur (Burr 1993; Mori and Burr 1993). Repetitive impulse loading causes the collagen of SCB to undergo fatigue leading to micro-fracture, callus formation, vascularisation and increased stiffness. Pathologic changes in SCB have been implicated in the pathogenesis of OA and have been found to be an early event in some animal models of OA (Layton et al. 1988). Joints such as the human shoulder joint (Muller-Gerbl 1998) and equine fetlock joint (Riggs and Boyde 1999) have been shown to demonstrate mineralisation patterns that are clues to the previous loading of the joint.

Increased mechanical loading of bone leads to sclerosis due to suppression of resorption (Frost 1983). Subchondral bone sclerosis is greater with OA due to increased number and reduced separation of trabeculae. However, material density is less due to the increased turnover of bone resulting in less highly mineralised bone and greater amounts of osteoid. Even though the material density of trabecular bone is less with OA, this is compensated for by the increased trabecular number and volume resulting in an overall stiffer structure (Burr 1998).

A possible mechanism for the initiation of articular cartilage damage may be the steep stiffness gradient in the SCB caused by healing of trabecular micro-fractures (Radin and Rose 1986) (Dequeker et al. 1995) causing fissures to form in the SCB. Varying properties of SCB could cause variations in the mechanical properties of articular cartilage (Mow and Soslowsky 1991). The temporal relationship of SCB sclerosis and articular cartilage degeneration is unknown, but it is widely recognised that SCB sclerosis is necessary for the progression of OA (Burr and Schaffler 1997; Burr 1998).

1.2.8 The equine metacarpophalangeal joint

The metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints, or fetlock joints, are formed by the articulation of the distal third metacarpal/tarsal bone (Mc3/Mt3), proximal proximal phalanx (P_p), and proximal sesamoid bones (PSB) (Figure 1.8). The hinge articulation, presence of the median sagittal ridge and strong collateral ligaments restrict motion almost completely to the sagittal plane. The centre of rotation passes through the proximal attachments of the collateral ligaments (Getty 1975).

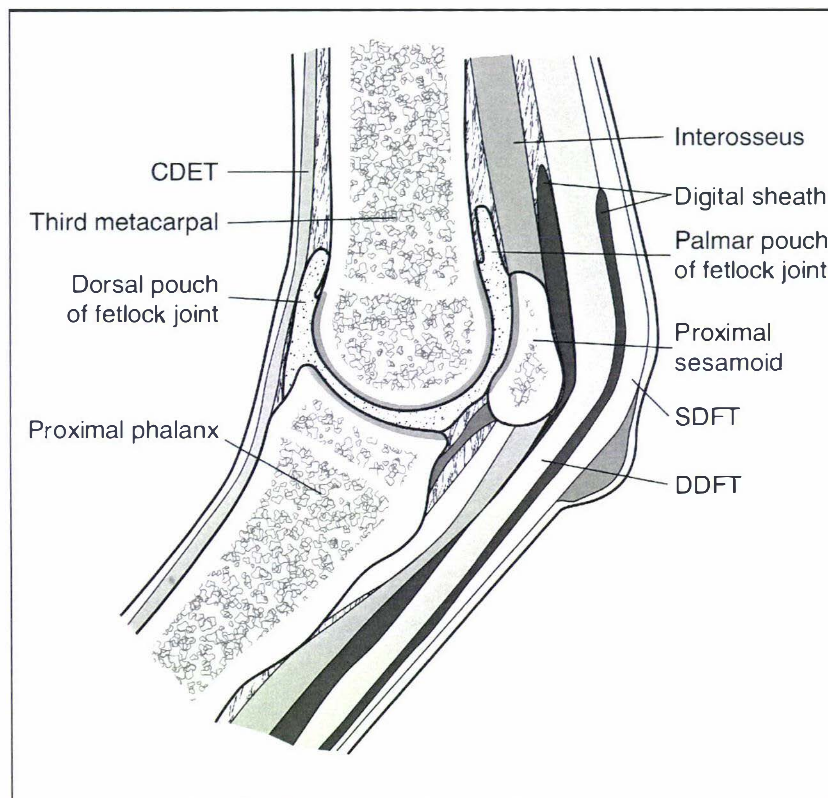


Figure 1.8: Diagram of a sagittal section of the metacarpophalangeal joint and the associated soft tissue structures.

Adapted from Equine Veterinary Journal (Strand et al. 1998).

The median sagittal ridge divides the distal Mc3 articular surface into a medial and lateral condyle with the medial having a larger surface area than the lateral. The transverse ridge is situated horizontally at the palmar aspect of the condyles. The joint surface of the first phalanx and the two sesamoid bones form a socket for the distal Mc3 and Mt3. The dorsal joint cavity of the fetlock joint extends 3-4 cm dorsoproximally under the digital extensor tendons, and the palmar pouch extends proximally to the bifurcation of the suspensory ligament. The latter

pouch can be palpated in the triangular space between Mc3/Mt3, the suspensory ligament and the sesamoid bones.

The sesamoid bones are embedded in the fibrocartilagenous intersesamoidean ligament. They are integral parts of the mainly ligamentous suspensory apparatus. The proximal suspensory apparatus consists of the suspensory ligament with the branches of the ligament inserting on the PSB. The distal suspensory apparatus consists of four short sesamoidean ligaments (straight, oblique, cruciate and short). The two collateral ligaments of the joint blend with and reinforce the joint capsule laterally (Nilsson and Olsson 1973). The collateral ligaments are partially divided into two layers: the superficial layer arises from the lateral or medial fossa of distal Mc3 and passes to the area distal to the articular margin of P_p, the shorter and stronger deep part arises in the fossa and passes obliquely and distally to insert on the abaxial surface of the PSB and proximal P_p (Getty 1975).

The MCP joint has the highest prevalence of site-specific degenerative and traumatic lesions of all joints (Pool 1996). The joint is extended during standing with a dorsal articular angle of approximately 140 degrees. With increased loading during the stance phase of high speed gaits the joint becomes hyper-extended. The susceptibility of the MCP joint to osteochondral disease in part relates to its small surface area, large range of motion and significant repetitive loading (Palmer and Bertone 1996). The full body weight of the horse is transmitted through the joint in mid-stance phase of the stride during galloping which results in the dorsoproximal margins of P_p contacting the dorsal articular margin of Mc3 and creating osteochondral damage (Palmer et al. 1996).

The sesamoidean articulation has also been proposed as one of the regions of the distal metacarpus under the most stress (Vilar 1995). The stress is thought to increase in response to repetitive cyclic trauma causing a proliferation of bone at the palmar region (Pool and Meagher 1990). Micro-damage of subchondral bone, osteocyte necrosis, osteoclast activity, haemorrhage and fibroplasia have been seen to occur in areas prone to parasagittal fracture of Mc3 condyles, and palmar condylar overload arthrosis (Kawcak et al. 1998; Norrdin et al. 1998).

An investigation into joint-specific contact areas under static loading of the proximal articular surface of P_p and Mc3 has aided the understanding of dorsoproximal P_p (Brama et al. 2001b) and dorsal Mc3 articular pathologic change. The contact areas and pressure distribution on P_p under static loading were determined using pressure film and methylene blue to quantify biomechanical stresses. Intermittent high loads were found to occur at the site of the

articulation of dorsoproximal P_p and the most dorsal extent of the articular margin of Mc3, and constant loads at the site of sesamoid articulation of palmar Mc3 (Brama et al. 2001b). Significant variation in the biochemical properties of the articular cartilage corresponded to the in-vitro measured biomechanical patterns of loading, reflecting the different functional demands. The constantly loaded palmar Mc3 region had the highest GAG content while the intermittently loaded dorsal region had higher collagen content and crosslinking (Brama et al. 2000).

Site-specific osteochondral pathology within the fetlock may be explained by the associated site-specific biochemical composition of the articular cartilage. The prevalence of osteochondral lesions at central proximal P_p, a continuously loaded site, are low, possibly because this tissue is better able to withstand biomechanical loading, as weight-bearing articular cartilage is known to respond better to increased loading than articular cartilage that is intermittently loaded or deprived of loading (Brama et al. 2001b). This is supported by the occurrence of pathology at the dorsal articular margin of P_p, a site only intermittently loaded during galloping and jumping but that during loading sustains loads 1.5 times greater than the central region and thus is more prone to injury. These findings strongly support a biomechanical role in the development of osteochondral disorders of the MCP joint.

Due to the heterogeneity of fetlock joint articular cartilage the location of sampling sites and age of the horse affect the outcome of biochemical studies (Brama et al. 2000; MacDonald 2002). For example, DNA and GAG content were higher at the palmar region of the distal Mc3 compared to the dorsal region (Brama et al. 2000)

Wear lines or score lines are the most common abnormality observed at post mortem examination in limbs of horses and occur independently of other joint lesions such as chip fractures. Wear lines are commonly found in the MCP joint and their aetiology and significance are uncertain. Light microscopy and ultrastructural observations of wear lines indicate that the chondrocytes of the tangential layer of the affected articular cartilage are necrotic and there is loss of proteoglycan as assessed by reduced safranin O staining of articular cartilage matrix (Pool 1996). A loss of orientation and density of collagen fibres separated by fluid was also seen by ultrastructural examination. These findings related to a loosening of the collagen framework as a result of exercise reported as strenuous, which involved exercising 6 days/week for 13 weeks (cantered at 8 m/sec for 4 weeks, 10 m/sec for 4 weeks for unknown times and distances, then galloped at 14m/sec over 4000m), in a typical New Zealand flat racing preparation programme (Brama et al. 2000a).

1.2.9 Biomechanical behaviour of articular cartilage and subchondral bone

1.2.9.1 Articular cartilage

The influence of mechanical loading on articular cartilage function and cellular metabolism had lead to the development of mathematical analyses to model the effects during loading (Lane Smith et al. 2000). Articular cartilage can be thought of as a biphasic material with a fluid and a solid phase, or as a triphasic material including an ionic component. Most of the fluid is free to move in and out of the tissue as a result of the loads applied. Water content determines the physical properties that enable articular cartilage to function effectively. The shape and organisation of proteoglycans is closely related to the water content (Gardner 1990).

Disruption of the normal joint architecture alters the flow-independent viscoelastic and equilibrium properties of articular cartilage (Schmidt et al. 1990). The biphasic theory, used by Mow and co-workers (Mow et al. 1984; Mow et al. 1989), predicts a loss of stiffness and increased permeability with loss of articular cartilage matrix components. The biphasic model has been modified to a triphasic theory to incorporate effects of osmotic pressure and electrostatic effects on total swelling behaviour of articular cartilage (Lai et al. 1991).

Since the amount of articular cartilage relative to SCB is small, articular cartilage contributes a minor share to peak force attenuation despite its greater efficiency. Increased articular cartilage thickness in areas of the joint surface experiencing high compressive hydrostatic stress have been predicted by Carter et al (Carter et al. 1987a; Carter et al. 1987b; Carter and Wong 1988; Carter 1990). Thinner articular cartilage is predicted at areas of lower hydrostatic compression and significant tensile force tangential to the joint surface. The longevity of articular cartilage, as for other bearing surfaces, is related to the amount of force and number of load cycles it sustains. Sparing articular cartilage from force is important in prolonging cartilage integrity (Radin and Paul 1971).

Mechanical injury is known to increase the risk of development of secondary OA and a number of in vitro models have been developed to study this (Patwari et al. 2001). As the biomechanical strain experienced by the tissue increases towards the upper end of the range, repeated minimal trauma causes tearing of collagen fibres, disruption of extra-cellular matrix, and submicroscopic fissures leading to remodelling and repair (Hurtig and Pool 1996).

Regional mechanical differences may influence articular cartilage metabolism. The response to shear stress of chondrocyte metabolism, and alteration of matrix molecular expression has been mostly investigated by in vitro application of shear stress on the tissue following deformation of

the matrix by the application of load in unconfined compression (Lane Smith et al. 2000). At low loading frequencies and low compression force, matrix synthesis is inhibited. In contrast, cellular metabolism is stimulated by higher compressive forces at the same frequency (Lee and Bader 1997; Knight 1997; Lee et al. 1998).

1.2.9.2 Subchondral bone

When a mechanical load is applied to a bone it deforms. The deformation is elastic and the bone will, within limits, return to its original shape unaltered once the load has been removed. If the degree of deformation rises above a threshold limit irreversible change may occur (Riggs 2002). Deformation arises from either a single extreme load or accumulation of repetitive lesser loads. Accumulated repetitive loads can result in fatigue damage. The degree of deformation is a function of the magnitude of the load and stiffness of the bone. Stiffness of the bone is determined by its geometry and material properties. The fatigue life (number of load cycles to failure) of every bone is logarithmically related to the extent of deformation caused at each cycle. The three dimensional structure and micro-structural characteristics of bone can change in response to the mechanical environment by the processes of modelling and remodelling. It is not known how long it takes in the horse for bone to complete adaptive modelling and remodelling following a significant change in its mechanical environment (Riggs 2002), however modelling of cancellous bone certainly occurs within 13 weeks of high intensity treadmill exercise (Firth et al. 1999).

1.2.10 Loading of articular cartilage and subchondral bone

The importance of weight-bearing for maintenance of healthy articular cartilage has been shown in a number of animal models and human clinical trials (Hall 1963; Thompson and Bassett 1970; Mankin and Lippiello 1970; Hock et al, 1983; Setton et al, 1998). All tissues have biomechanical set points defining a window of usage within a physiologic range (Jee 1991). Immobilisation or reduced loading leads to a decrease in glycosaminoglycan (GAG) synthesis and content in human, canine and equine articular cartilage (Kiviranta et al. 1987; Sah et al. 1989; Richardson and Clark 1993; Burton-Wurster et al. 1993; Kiviranta et al. 1994; Kim et al. 1994; Kaab et al. 2000; Malinin and Ouellette 2000; Leroux et al. 2001). Increased dynamic loading within physiologic ranges, as occurs during the stance phase of the gallop, causes an increase in GAG synthesis and content (Jones et al. 1982; Kiviranta et al. 1987; Gray et al. 1988; Sah et al. 1989; Sah et al. 1991; van den Hoogen et al. 1999; Malinin and Ouellette 2000). Persistent increased joint use may also alter the composition and organisation of the matrix but

this has not been clearly demonstrated in the skeletally mature individual (Buckwalter and Hunziker 1999).

Articular cartilage undergoes a two phase (viscoelastic) deformation under load (Kempson 1979; Mow et al. 1980). Initially water moves rapidly from the matrix and collagen compresses causing instant deformation. Then time dependent compression, or the creep phase, occurs where water moves more slowly through the matrix as the load is shifted to the solid component of the articular cartilage. Normal synovial joint function depends on the load-bearing properties of the extra-cellular matrix (Kempson 1979; Mow and Rosenwasser 1987).

The ability of articular cartilage to withstand the local conditions depends on its biochemical composition (Buckwalter et al. 1987; Heinegard and Sommarin 1987; Heinegard and Paulsson 1987). Normal articular cartilage, although rarely >1mm thick, is designed to withstand compression and shear, operating under large variations in load and sliding speed. Its stiffness and resilience in compression leads some authors to attribute it with an ability to distribute loads to minimise peak stresses on subchondral bone (Kempson 1979; Mow and Rosenwasser 1987).

Joint loading and motion has been shown to induce a wide range of metabolic responses in articular cartilage (D'Lima et al. 2001b). When articular cartilage is stretched in tension the forces are predominantly resisted by the collagen fibrils. The tensile modulus or stiffness are measures of the resistance to tensile loading and depend on the density, orientation and cross linking of the collagen fibres. Compression is characterised by viscoelastic characteristics and redistribution of fluid within the articular cartilage. Resistance to compression is influenced by PG content. Shear forces result in both stretching and deformation of the articular cartilage. Collagens are primarily involved in resisting of shear (Maroudas 1976) with a contribution from PG to improve effectiveness of coping with shear forces (Setton et al. 1999).

Chondrocyte metabolism is affected by mechanical loading (Little et al. 1996; Wong et al. 1997; Lee and Bader 1997). The signals from the changing chondrocyte/matrix physio-chemical environment and the cellular disturbances occurring in OA, are poorly understood (Hall and Bush 2001). Mechanical load can influence the material properties of articular cartilage, principally by altering the proteoglycan concentration. In normal joints the load-bearing areas are thicker, have a higher proteoglycan concentration and are mechanically stronger than non-load-bearing regions in the same joint (Urban 1994).

Supra-physiological static or impact loading causes articular cartilage deterioration (Repo and Finlay 1977). A load-induced shift in the metabolic balance between articular cartilage

formation and degradation is associated with matrix destruction and the appearance of osteoarthritic changes (Shuckett and Malemud 1989). Proteoglycan release has been used as a measure to estimate the extent of in vitro mechanical injury and validates the premise that in vitro loading at the selected injury levels, produces metabolic response indicative of articular cartilage injury (D'Lima et al. 2001b).

Neonatal equines appear to be born with a biochemically 'blank' joint that develops topographical variety of the mature joint in the first few months of life. This heterogeneity is most likely initiated by weight-bearing and forces acting across the articulation (Brama et al. 2000b). These adaptive responses of articular cartilage to loading may be mechanisms to reduce articular cartilage damage at heavily loaded sites (Brama et al. 2000). Variations of load occur within and between joints and can be categorised into low level constant load such as during standing, intermittent load during locomotion, and high load during training or racing (Palmer and Bertone 1996; Brama et al. 2000b).

The mechanism of responding to mechanical loading and modifying the tissue is through cellular detection of strains or matrix alterations (Buckwalter and Grodzinsky 1999). When loaded, articular contact increases, this distributes load, decreases stress and increases joint stability. Due to its limited thickness the ability of articular cartilage to act as a shock absorber is limited and at a certain point it must transmit the load to the subchondral tissues which at physiological loads remodel to provide maximum strength and absorption capacity (Pugh et al. 1973a).

Chondrocytes react to their mechanical environment with changes in cell shape and volume that coincide with modulation in metabolic activity (Kim et al. 1995; Guilak et al. 1995; Bird et al. 2000b). The mechanism(s) by which chondrocytes 'sense' and respond to changes in their physiochemical environment are poorly understood (Urban et al. 1993). Deformation of the cell wall directly affects the transport of solute, assembly of proteins and other cell functions (Alberts et al. 1989).

With the development of morphometric techniques it is possible to detect evidence of the functional response of the cells to mechanical, physicochemical and biological stimuli (Stockwell 1990). Changes in matrix hydration during physiological and pathophysiological loads can lead to changes in cell volume that may alter matrix metabolism (Bush and Hall 2001b). A number of chondrocytes have short processes or microvilli extending from the cell into the matrix. These microvilli may have a role in sensing mechanical changes in the matrix. Cell membrane receptors (integrins), are sensitive to the biomechanical environment. Injurious

compression on chondrocytes affects turnover and release of proteoglycans (Quinn et al. 1998) leading to loss of normal articular cartilage matrix homeostasis (Mankin et al. 1971). Chondrocytes in each of the articular cartilage zones may behave differently in response to applied load. The superficial zone of articular cartilage has been shown to be important in preserving normal compressive behaviour of the articular cartilage and normal weight-bearing of the joint (Setton et al. 1993). Differences in fibronectin synthesis between superficial and deep zone chondrocytes have been demonstrated in both bovine and human articular cartilage (Hayashi et al. 1996).

Applied compressive stresses above 25 MPa kills most chondrocytes in articular cartilage. Between these extremes of loading (2-25MPa) little is known about the chondrocyte responses to compressive stress (Clements et al. 2001). This range probably encompasses the effects of vigorous physical activity. The full physiological range of joint loading in vivo is unknown and may vary between joints and species. In humans, maximum compressive stresses for human hip joints are 5-8 MPa during level walking (von Eisenhart et al. 1999). The limited anabolic response of chondrocytes under loading has meant that autogenous chondrocyte grafts used in human orthopaedics have ambiguous long term results (Messner and Gillquist 1996).

Loads on the appendicular skeleton of the horse during locomotion show a linear relationship with speed (Riggs 2002). Subchondral bone density and strength also adapts to imposed mechanical forces (Kawcak et al. 2001). The increase in density may be more marked in animals that have undergone intense training (Riggs et al. 1999b). Previous studies have shown that subchondral bone in the equine third carpal bone (Young et al. 1991b) and the palmar/plantar aspects of Mc3 is more dense than other areas in the associated joints (Pool and Meagher 1990; Riggs et al. 1999b).

In humans the loss of the ability to use precise neuromuscular corrections to reduce joint loading has been described and is termed 'micro-klutziness'. Loss of deceleration prior to heel strike, due to fatigue or inherent in-coordination, causes subtle repetitive impulsive loading, often resulting in knee joint pain. Minor neuromuscular insufficiencies during joint loading may lead to degenerative changes in articular cartilage and subchondral bone (Ito et al. 1990). Repetitive impulsive loading has been shown to create subchondral bone sclerosis leading to osteoarthritis in rabbits (Radin et al. 1991). Micro-klutziness has not been investigated in the horse but if similar subtle micro in-coordination occurs, it is possible that fatigued or neuromuscularly challenged Thoroughbred racehorses are prone to SCB micro-injury and sclerosis secondary to reduced ability to attenuate load during galloping (Radin 1999).

1.2.11 Osteoarthritis (OA)

Millions of people throughout the world are affected by osteoarthritis (Lohmander 2000). OA is a significant cause of lameness and disability in humans, horses, dogs and other domestic species. Approximately 85% of the human population are affected by OA by age 75 (Sack 1995). The increasing prevalence of joint disease in the human population has resulted in an increased social and economic burden. In the USA the estimated cost of OA is $15.5 billion per annum (Yelin 1998).

OA, also termed degenerative joint disease, is a group of disorders manifested by a continuum of progressive degenerative changes of articular cartilage, remodelling of subchondral bone, and inflammation of the synovium and associated structures (Pelletier et al. 1985; Howell 1986; McIlwraith and Vachon 1988; Frisbie et al. 1997). Articular cartilage exhibits manifestations characteristic of OA with inappropriate mechanical loading of the joint, traumatic or exercise-induced damage, ligament instability, bony misalignment or excessive weight-bearing, or following joint sepsis.

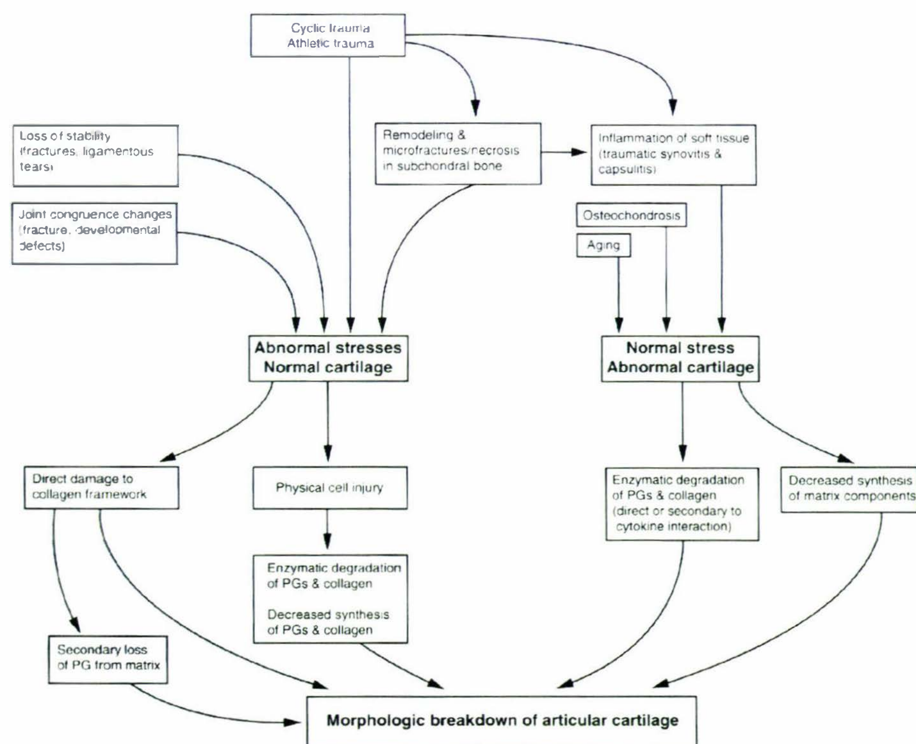


Figure 1.9: Diagram of the factors involved in articular cartilage degradation in osteoarthritis.

From Joint Disease in the Horse, W.B. Saunders Company, Philadelphia (McIlwraith 1996)

Breakdown of articular cartilage in OA involves degeneration of the extra-cellular matrix macromolecules and decreased expression of chondrocyte proteins necessary for normal joint function (Figure 1.10). Initially there is loss of aggregating PG from the matrix with secondary collagen degradation, evident as surface fibrillation (Mankin and Lippiello 1971; Maroudas et al. 1985; Curtin and Reville 1995).

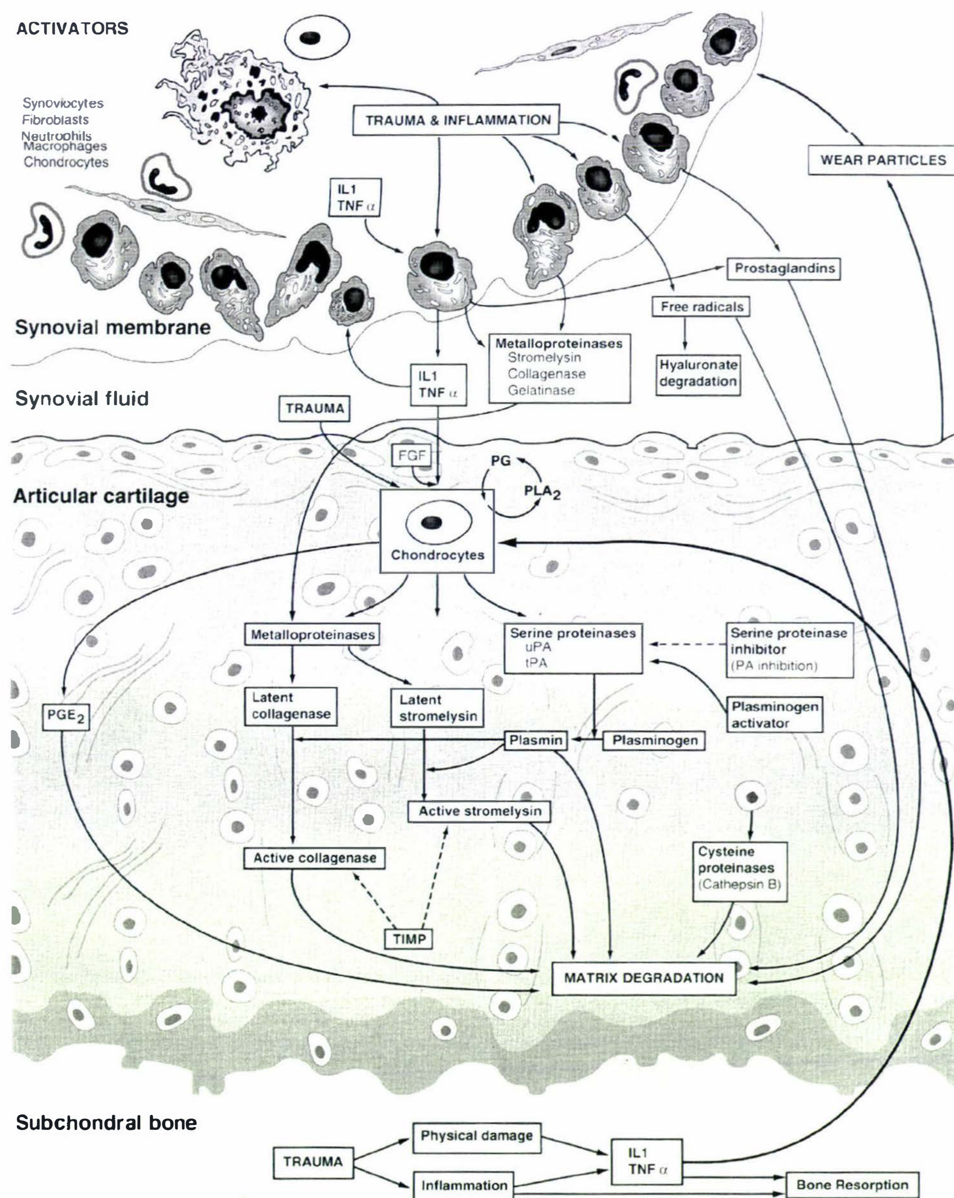


Figure 1.10: Diagram of factors involved in enzymatic degradation of articular cartilage matrix.

Il-1 = interleukin-1; TNF α = tumour necrosis factor α; FGF = fibroblast growth factor; PG = prostaglandin; PLA$_2$ = phospholipase A$_2$; uPA = urokinase plasminogen activator; tPA = tissue plasminogen activator; PA = plasminogen activator; PGE$_2$ = prostaglandin E$_2$; TIMP = tissue inhibitor of metalloproteinase. ***From Joint Disease in the Horse, W.B. Saunders Company, Philadelphia (McIlwraith 1996)***

Degenerative changes that occur in joint tissues of patients with OA are distinct from those that occur in the non-osteoarthritic population during aging (Burr 1998). The pathogenesis of OA has been described as an attempt at healing (Radin 1995). It is well reported that mature articular cartilage has a poor intrinsic ability to repair (Tew et al. 2001; Colwell et al. 2001). However, during degenerative change or injury, articular cartilage can respond in a reparative fashion. Anabolic events include matrix synthesis and renewed chondrocyte proliferation (Archer 1994; Henrotin and Reginster 1999). Unfortunately anabolic responses are usually insufficient to repair even partial lesions, particularly under functional loading. Osteoarthritic cartilage often exhibits increased amounts of Type I collagen and synthesis of proteoglycans characteristic of immature articular cartilage. The shift in articular cartilage phenotype in response to OA yields a matrix that fails to support normal joint function (Lane Smith et al. 2000).

There is consensus that OA is characterised by subchondral sclerosis and varying degrees of articular cartilage damage. Disagreement exists about whether subchondral bone changes are concurrent with, primary to, or secondary to articular cartilage degeneration (Burr and Schaffler 1997). It may be that to different degrees both occur simultaneously. Although focal articular cartilage damage is accepted to be the typical characteristic in OA, subchondral calcified tissues have been implicated in exacerbating the disease (Radin and Rose 1986). In early studies by Radin and co-workers (Simon et al. 1972; Radin et al. 1973) and in a more recent macaque model of OA (Carlson et al. 1994), subchondral bone sclerosis preceded articular cartilage changes (Fuller et al. 2001)

Joint inflammation is likely to result from local proinflammatory cytokine-induced degeneration, but the synovial inflammation of OA is less severe than immune-mediated joint diseases, such as rheumatoid arthritis (RA). Inflammation onset may be earliest in post–traumatic OA, in which activated synovial tissue rather than articular cartilage is the primary producer of inflammatory mediators.

Chondrocyte death (apoptosis or necrosis) is a feature of, and may contribute to, the progression and pathogenesis of osteoarthritis (OA). Chondrocyte apoptosis occurs in human and experimentally induced osteoarthritis, and is positively correlated with the severity of articular cartilage destruction (Lotz et al. 1999). Chondrocyte apoptosis and articular cartilage matrix degradation are linked to mechanical events in the pathogenesis of OA (Lotz et al. 1999). There is evidence to suggest that OA begins at the surface of the articular cartilage (Mitchell et al. 1992).

In human articular cartilage the calcified cartilage and subchondral bone plate form a 'mineralisation front' (Imhof et al. 1997), which has been reported to advance into, and progressively calcify the non-mineralised articular cartilage in OA (Oegema et al. 1997). This is in contrast to equine articular cartilage in which the calcified cartilage zone has been reported to increase in thickness in OA without a decrease in the thickness of the non-calcified cartilage (Murray et al. 1999).

The diagnosis of OA and therapeutic recommendations have been thoroughly reviewed in both man and the horse (Clegg et al. 1997; Kidd 2001). Early detection using imaging equipment and molecular techniques may allow therapeutic intervention before the disease process becomes irreversible (Fuller et al. 2001). Molecular markers such as proteoglycan fragments generated by MMPs, collagen propeptides, cartilage oligomatrix protein (COMP), bone morphogenic protein (BMP) and bone sialoprotein (BSP), to non-invasively detect early changes in articular cartilage and bone are being validated (Vignon et al. 2001). Recent work suggests that biomarkers will be useful in differentiating exercise-induced changes from OA pathologic changes (Frisbie 2002).

Current clinical therapies utilise anti-inflammatory agents to reduce inflammatory mediator-induced damage to joint tissues (McIlwraith and Vachon 1988; Frisbie et al. 1997). More recent developments in therapy have investigated use of agents such as insulin like growth factor-1 (IGF-1) to increase matrix metabolism above basal equilibrium (Frisbie et al. 2000). Gene therapy has been investigated for the modification of cellular expression of cytokines (Saxer et al. 2001).

Reduction of inflammatory mediators through disease-modifying processes may help protect joint tissues from initial insult. In a study into use of gene therapy in horses, interleukin-1 receptor antagonist (IL-1Ra), a molecule with anti-arthritic potential, was expressed in diseased joints after intra-articular administration of an adenoviral gene transfer vector (Ad-IL-1Ra) carrying the equine IL-1Ra gene sequence. Interleukin-1 (IL-1) from macrophages, and other cells producing antigen, is active in the inflammatory process. Findings suggested that IL-1Ra had a protective effect on proteoglycan loss in an osteochondral fragment model of joint inflammation. This treatment has potential as a viable therapeutic option for the equine patient and encourages development of future anti-arthritic gene sequences (Frisbie and McIlwraith 2000).

OA may be influenced by a number of environmental, hormonal, intrinsic and extrinsic factors within an individual. It has recently been reported to have a large genetic component in humans

(Reginato and Olsen 2002). Studies to investigate gene-gene and gene-environment interactions are necessary in animal models to better understand the pathogenesis and provide earlier preventative strategies and treatments. Chondrocyte metabolic activity at the early stage of OA is different from that associated with established disease, and therefore different therapeutic strategies will be needed depending on the stage of the disease process (Henrotin and Reginster 1999). The use of pluripotential stem cells (Jorgensen et al. 2001), biomaterials, bioactive molecule and gene transfer technology are emerging as tools in tissue bioregeneration and may have application for restoring joint function in articular cartilage loss (Schultz et al. 2000). As micro-structural molecular interactions are investigated the OA processes may be able to be better modulated therapeutically with the goal of effective chondrocyte modulated repair and regeneration of articular cartilage (Stockwell 1990).

In most cases the joint disease process in humans and horses results in inevitable damage of the joint and joint associated tissues. In Thoroughbred racehorses this leads to increased training days lost, increased treatment costs and premature retirement from athletic function. Development of techniques to assess articular cartilage and bone will help accurately identify those horses at risk of OA, as well as the success of therapies.

1.2.12 Methods of assessing articular cartilage and bone

There is a wide array of in vitro techniques for assessing human and equine articular cartilage and subchondral bone. Unfortunately early detection and differentiation between physiological and pathological changes within the joint in vivo is difficult. The development of non-invasive or minimally invasive methods of evaluating articular cartilage and bone for early signs of OA will contribute to a more thorough understanding of the events leading to clinical OA. In order to maximise the potential of any therapeutic intervention in OA it is essential to diagnose the disease at an early stage (Fuller et al. 2001).

1.2.12.1 In vivo

Clinical observation and physical examination can identify signs such as pain, effusion, and loss or reduction in function that are suggestive of joint pathology. Plain and high resolution, and more recently digital, radiography and microradiography are widely used to evaluate changes in SCB. As well as superimposition of structures obscuring important findings, standard radiographs only detect bone loss exceeding 30% (Carrig 1997), as such, techniques are reliable only once the disease process is well established. The present diagnostic aids to investigate OA

are not sensitive until the disease is progressed to macroscopic articular changes seen at arthroscopy or bony changes able to be detected on radiographs.

Arthroscopy because of its wide and moveable field, clarity and magnification, allows direct visualisation of the articular cartilage and has been the gold standard for assessing articular cartilage lesions in vivo (Fife et al. 1991). The correlation of arthroscopy scoring of articular cartilage with radiographic changes in the veterinary literature has been variable, from poor in equine carpal OA (Kannegieter and Burbidge 1990) to good in the equine stifle (Steinheimer 1995).

MRI yields outstanding tissue contrast and anatomic definition (depending on the acquisition sequence), and shows most promise as a technique for detecting early articular cartilage degeneration (Tucker and Sande 2001). It uses the magnetic properties of tissues exposed to a strong magnetic field, and associated radiofrequency pulse to characterise each tissues magnetic characteristics (Tucker and Sande 2001). MRI is becoming the imaging method of choice for articular cartilage as it is less invasive than arthroscopy (Chung et al. 2001).

Ultrasound can directly image the articular cartilage surface and synovium (Carrig 1997). Ultrasonic techniques have been developed to characterise articular cartilage surface fibrillation and synovial thickening directly (Chiang et al. 1997).

Progress in the examination of bone in vivo has been accelerated by clinical research into human osteoporosis, where accurate precise and safe methods of assessing bone turnover were required (Lepage et al. 2001). Imaging techniques are used to detect subchondral bone sclerosis in OA. Scintigraphy can image a number of joints in a single examination and can be predictive of the radiographic progression of OA. However it is non-specific and lacks spatial resolution (Carrig 1997). Quantitative ultrasonography (QUS) has been used for assessing equine bone. It uses bone speed of sound measurements using axial transmission mode along the cortex giving information stiffness, architecture, porosity and bone mass (Lepage et al. 2001).

Bone mass is measured by bone mineral density (BMD). Bone mineral density is expressed as grams of calcium per square centimetre of bone cross section (gm/cm^2) and in grams per cubic centimetre (gm/cm^3) in three dimensional techniques (Lepage et al. 2001). Computed tomography (CT) spatially separates overlying tissues and provides high contrast between calcified structures and soft tissues but the contrast is inferior to that of magnetic resonance imaging (MRI) (Carrig 1997), and it has its main application for calcified tissues. Radiation based techniques such as single or dual photon x-ray absorptiometry (gm/cm^2) and radiographic

absorptiometry (mm Al) have been used in people to assess bone healing and in pathologic bone conditions such as osteoporosis to give an estimate of BMD (Markel and Chao 1993; Davidson et al. 1996). These techniques have logistical limitations for use in live horses as do CT and MRI.

Biochemical markers as indicators and monitors of articular cartilage degeneration in early OA have been investigated (Sharif et al. 1995; Sharif et al. 1995a; Sharif et al. 1995b; Sharif et al. 1996), and shown to be associated with disease activity and outcome. Molecular markers may enable early and non-invasive detection of traumatic OA. Biochemical or immunological assays detects these molecules released into synovial fluid and then blood and urine. The ideal marker of articular cartilage degeneration is one that is not found in normal articular cartilage or other tissue. The marker must be able to be validated against a gold standard which in the case of joint disease is usually radiography or magnetic resonance imaging (MRI). Since the degeneration of articular cartilage has long been accepted as the pivotal diagnostic feature of OA, correlation between the degree of articular cartilage damage and the change in concentration of any biochemical marker from normal in serum or synovial fluid will give criterion validity to that marker.

Serum biochemical markers also show promise in assessment of bone in vivo. Detecting biochemicals in bone, in particular markers indicating bone formation such as bone specific alkaline phosphatase (BAP) and bone degradation e.g.; hydroxyproline (HOP), have shown validity on their potential for use as non invasive diagnostic aids in equine OA (Lepage et al. 2001). A positive correlation between BAP and articular cartilage damage suggests that there is a link between bone turnover and articular cartilage damage in OA (Fuller et al. 2001).

1.2.12.2 Ex vivo

Macroscopic observation at necropsy is initially used to assess synovial joints for evidence of pathology. Evaluation of the joint surface for defects such as wear lines or fibrillation can be facilitated with application of particulate dye, such as Indian ink (Cantley et al. 1999), which has been improved using image analysis techniques. The mechanical properties of articular cartilage can be evaluated by testing of in situ cartilage or cartilage explant under differing loading conditions

Light microscopy is most commonly used to assess articular cartilage and bone morphology, with a number of similar techniques. The microscopic structure of normal articular cartilage

and naturally acquired OA has been reported to be identical in horses and people (Morris and Treadwell 1994).

Haematoxylin and Eosin (H&E) is one of the most widely used stains to examine chondrocyte morphology and articular cartilage structural integrity (Hyllested et al. 2002). The Mankin scoring system was devised to classify the severity of human hip OA (Mankin et al. 1971). The Modified Mankin scoring system, currently accepted as a method to assess equine articular cartilage sections for evidence of OA (Foland et al. 1994; Frisbie et al. 1997), semi-quantitatively grades structural changes and cellular density in a section of accurately known thickness.

Biochemical tests such as DNA content, GAG synthesis and content, and collagen content quantify biochemical components of articular cartilage or bone. Staining techniques used for assessment of PGs and GAGs, include Safranin O/Fast green, Alcian blue and Toluidine blue. Evidence from human and veterinary literature shows that proteoglycan staining is not completely reliable, particularly at low substrate concentrations. Safranin O and Toluidine blue are the most reliable GAG and PG stains when GAG depletion is not severe (Hyllested et al. 2002). The stoichiometric staining characteristics of Toluidine Blue in the detection of articular cartilage GAG have been compared to those of Safranin O and found to be inferior (Poole 1970; Rosenberg 1971). Toluidine blue has been used for qualitative assessment of equine articular cartilage (Todhunter 1996).

The use of Safranin O as a marker for GAG depletion in articular cartilage disease such as OA has been described (Rosenberg 1971; Hyllested et al. 2002); loss of Safranin O staining suggests reduced PG within articular cartilage(Foland et al. 1994; Frisbie et al. 1997). Safranin O/Fast Green (SOFG) is the most widely used stain combination for GAG and proteoglycan content.

Damage to the collagen network can be detected microscopically. Polarised light and birefringence using picrosirius red stain has been used to assess collagen fibrils (Mollenhauer et al. 2002), which lose their normally birefringent pattern and organisation in damaged matrix.

Electron microscopy (scanning and transmission), particularly environmental scanning electron microscopy (ESEM), is useful as it allows the surface of articular cartilage to be imaged without the need for fixation (Graindorge and Stachowiak 2000).

Confocal microscopy has been used to assess articular cartilage and bone and more recently chondrocyte viability using fresh tissue. Methods that allow assessment of fresh tissue provide a more accurate indication of the status of the cells.

1.2.13 Effect of exercise on articular cartilage and subchondral bone

1.2.13.1 Articular cartilage

During the neonatal period musculoskeletal tissues go through a rapid period of growth and modelling, which in the horse, is influenced strongly by weight-bearing (van Weeren 2000). The biochemical composition of tissues is directly related to biomechanical function. Heterogeneity of proteoglycan and chondrocyte metabolism have been shown to develop postnatally as a functional adaptation to weight-bearing in sheep (Little and Ghosh 1997) and horses (Brama et al. 2000b).

A certain amount of exercise is essential for the process of developing biochemical heterogeneity in young horses. It is possible that biomechanical loading of the biochemically uniform joint early in life affects final PG and collagen composition. This in turn may influence articular cartilage composition and structural integrity and future resistance to injury (Brama et al. 2000b). A delay in the development of the collagen framework may cause lasting debility in the tissue. The process of achieving biochemical heterogeneity, normally completed by five months of age, is delayed in those animals deprived of exercise (Brama et al. 2002b). Brama has stated that 'limited and well dosed loading of the articular cartilage by specific training in the first few months of life may improve the final quality of the tissue' (Brama et al. 2002b).

The quantity and intensity of exercise required for appropriate development of articular cartilage is as yet unknown. Articular cartilage composition within a joint is likely to be dependent on local variation of loading within the joint and the amount the joint is used. Thus, exercise would be expected to alter articular cartilage composition. However, the effect of exercise on articular cartilage composition and development of pathologic change is not clear (Murray et al. 2001). In the carpus exercise and loading-related changes occur in the macromolecular characteristics of the matrix (Murray et al. 2001).

Moderate exercise, involving stall confinement and training 6 days/week with 12-24 40m sprints until five months of age, then 32 and 16 sprints on alternate days, with 0.5 hour of free exercise after sprints, has shown beneficial effect on articular cartilage of young horses (Brama et al. 1999b) however this has not been the case for strenuous exercise. In a study involving

seven two year old Thoroughbred horses subjected to strenuous exercise (exercising 6 days/week for 13 weeks (cantered at 8 m/sec for 4 weeks, 10 m/sec for 4 weeks for unknown times and distances, then galloped at 14m/sec over 4000m), in a typical New Zealand flat racing preparation programme), compared to 7 non-exercised controls, it was found that gross lesions of the MCP joint were more severe in the exercised than control groups and significant alterations in the collagen network were present in the exercised horses and physiological biochemical heterogeneity was absent (Brama et al. 2000a).

After 17 weeks of strenuous exercise, Standardbred equine dorsal third carpal bones have been shown to exhibit topographical variation in chondrocyte synthesis of proteoglycans which may be related to variations in mechanical loading (Little et al. 1997; Little and Ghosh 1997). The equine middle carpal joint has shown topographical variations in articular cartilage mechanical properties which related to exercise and superficial loss of matrix proteoglycan content at sites of clinical articular cartilage lesions. The dorsal margins of the third and intermediate carpal bones, sites commonly affected by lesions, showed evidence of early OA, including fibrillation and decreased PG, in strenuously exercised horses compared to those undergoing gentle exercise (Murray et al. 2000). This corresponds to the findings in the dorsal articular margins of the fetlock joint which are intermittently loaded and also had reduced proteoglycan content when compared to more constantly loaded sites (Brama et al. 2000).

Reports on the effect of exercise on articular cartilage components such as PG are variable with both anabolic (Parkkinen et al. 1992; Malinin and Ouellette 2000) and catabolic (Videman and Eronen 1984; Malinin and Ouellette 2000) effects reported. The differences in the studies may be explained due to age, species, breed, and intensity and duration of the physical activity. There is no correlation with age for the content of collagen or its crosslinks in mature equine (age range 3-40 years) (Brama et al. 1999a) or human articular cartilage (Bank et al. 1998). This relates to the slow turnover of collagen in adult articular cartilage. Estimated to be 120 years in canine and 350 years in the adult human (Brama et al. 2000b). The slow turnover of collagen in adult articular cartilage was not present in the neonatal animal where collagen content, hydroxylysine and hydroxylyslpyridinoline (HP) crosslinks all changed with age (Brama et al. 2000b).

Articular cartilage from the MCP joint has been shown to adapt to intensive training (Bird et al. 2000b) with increased synthesis of PG, however site differences were not investigated and the sample sites within the fetlock joint were not described and so topographical differences might have affected the results (Brama et al. 2000). In a recent study investigating the effect of exercise on developing musculoskeletal tissues 43 Dutch Warmblood foals were divided into

three groups. One group was withheld exercise and stall confined from birth to 5 months of age, the second group was housed under the same conditions but underwent an exercise programme and the third was given free pasture exercise as a control. A number of foals from each of the three groups were kept under a similar exercise programme for an additional 6 months to determine if any of the observed effects in the musculoskeletal tissues were reversible (van Weeren 2000). Differences in articular cartilage composition found between exercised groups of foals aged 5 months and 11 months, reflected changes in extra-cellular matrix of articular cartilage during this period of rapid growth. Older foals had higher tensile strength of articular cartilage and a more tightly organised matrix. Basal metabolism, as evidenced by ^{35}S uptake, was not significantly different between groups. However when chondrocytes were serum stimulated, maximal absolute values in the 11 month group were lower than the 5 month indicating that maximal synthetic capacity had substantially decreased in the older group.

Withholding exercise influenced the GAG content, which was increased with free paddock exercise. The GAG levels of all three groups were similar at 11 months (6 months after cessation of the different exercise conditions) (Brama et al. 1999b). There was a significantly higher basal metabolic rate of the chondrocytes in the exercised group when compared to the other two groups (van den Hoogen et al. 1999). From this study the basal metabolism of all three exercise groups was similar but the chondrocytes from the trained group could not be further stimulated.

1.2.13.2 Subchondral bone

Differences in exercise in the first few months of life have a significant effect on the components of bone (van Weeren 2000). It is not clear whether exercise has a beneficial or harmful effect on immature subchondral bone.

The physiological or pathological effect of exercise on SCB is controversial but most likely depends on the type of exercise, site studied, and species (Kawcak et al. 2001). Adaptive responses have been demonstrated in third carpal bones (Young et al. 1991b; Firth et al. 1999) and proximal sesamoid bones (Young et al. 1991a) of exercised horses. The biochemical composition of subchondral bone is affected by exercise (Brama et al. 2002a).

Pathological findings of subchondral bone of racehorses shows evidence of increased density in response to exercise (Pool and Meagher 1990). The effects of exercise and training have been associated with osteochondral pathologic change in the horse (Murray et al. 2002). The effect

of exercise of increasing bone mineral density particularly in immature individuals has been demonstrated (Cornelissen et al. 1999; Firth et al. 1999). Studies in the Thoroughbred racehorse show modification of the geometric properties of the distal third metacarpal bone in response to training (Riggs 2002). Biomechanically tested specimens from Mc3 of exercised horses had a significantly higher impact strength compared to those from non-exercised controls (Reilly et al. 1997). Firth et al demonstrated local adaptive response of increased bone mineral density in the radial, intermediate and third carpal bones of intensely exercised horses (Firth et al. 1999). Galloping exercise has also been shown to result in localised subchondral sclerosis in the palmar region of distal Mc3 (Firth, 2001). The presumed increase in sclerosis appears to be an adaptive response to exercise, but has been associated with overlying articular cartilage pathologic change, and occurs at common sites of osteochondral injury (Murray et al. 1999)

Cumulative micro-damage leading to fatigue fracture has been reported in a number of athletic species including human athletes and dancers, racing greyhounds and horses (Johnson et al. 2000; Johnson et al. 2001). Adaptive site-specific bone remodelling is thought to be induced by cyclic asymmetric loading as is encountered in central tarsal bone fracture in greyhounds raced on circular tracks (Johnson et al. 2000). As in other species, exercise intensity and duration influences the effect on equine joint tissues particularly subchondral bone (Kawcak et al. 2001).

There is evidence that the mechanical features of bone are related to the mineral content and biochemistry of the collagen network. Exercise affects the enzymatic post-translational modifications of the collagen component of SCB (Brama et al. 2001a), and there is large topographical variation in histological characteristics of subchondral bone in the ovine femoropatellar joint, most likely representing adaptation to different functional demands within the joint (Armstrong et al. 1995). Withholding physical activity in horses less than 5 months of age affects the biochemical characteristics of subchondral bone, but only at sites of maximum stress during physical exercise (Brama et al. 2001a).

The time a horse requires in training before the bone adaptive response is complete is unknown (Riggs 2002). It is possible to train bone to reduce the likelihood of fatigue related injury. Training regimes have been recommended to be carefully targeted for the type of work the animal will be performing. Significant changes in peak strain were seen when speed was increased from trot (5.5m/s) to gallop (16.6m/s). Training for prolonged intervals at slower speeds will induce a modelling response which is inappropriate for racing speeds, and short interval high-speed work can stimulate an appropriate modelling response with minimal risk of fatigue damage (Riggs 2002). In a group of yearling Thoroughbred horses there was found to be significantly less remodelling of the Mc3 in exercised group compared to non-exercised

controls; remodelling in ribs was similar in both groups (McCarthy and Jeffcott 1992). Therefore it has been proposed by Riggs that inhibition of remodelling may occur under high strain rates and that once the strain is removed or reduced then remodelling can occur in a relatively 'safe' environment (Riggs 2002).

Macro-morphological and micro-morphological study demonstrated characteristic sclerosis patterns in distal Mc3 subchondral bone of Thoroughbred racehorses. The palmar aspect of the condyles was significantly more dense than that of the sagittal ridge (Riggs et al. 1999b), associated with the predicted distribution of loads on the articular surface. This adaptive response was hypothesised to result in a stiffness gradient and stress concentration at the axial margin of the condylar bone. Which may initially affect articular cartilage and result in osteochondral damage if not complete parasagittal fracture (Riggs 2002).

1.2.13.3 Exercise and Osteoarthritis

Moderate regular exercise may not influence the development of OA, however high levels of repetitive impact may increase the risk of articular cartilage damage (Buckwalter and Lane 1997). Moderate level training did not increase the development of OA in adults aged over 50 years (Panush et al. 1986; Lane et al. 1993; Lane and Buckwalter 1993), but high level physical exercise did predispose to OA in men aged under 50 (Cheng et al. 2000). A high level of physical activity appeared to be a moderate risk factor for severe hip OA in women (Vingard et al. 1998). Experimentally, a lifetime of regular moderate exercise, treadmill walking at 3 km/hr for 75 minutes, 5 days a week for 527 weeks while carrying jackets weighing 130% of their body weight, has not been associated with OA in dogs (Kiviranta et al. 1988; Newton et al. 1997).

In contrast, strenuous training over 18 months was associated with site-specific changes in articular cartilage composition and PG depletion in canine and mouse articular cartilage consistent with increased OA in exercised compared to control subjects (Lapvetelainen et al. 1995). It appears that the ability of articular cartilage to cope with exercise may be determined by exercise intensity (Murray et al. 2001). Age-related OA occurs naturally in horses (Cantley et al. 1999). It is hypothesised that the joint stresses incurred during training and racing would lead to an acceleration of these changes (Cantley et al. 1999).

In people and animals, certain activities are associated with increases in OA in particular joints. For example hip and shoulder OA in sled dogs (Millis and Levine 1997), and carpus and fetlock

OA in Thoroughbred racehorses (Pool 1996; Murray et al. 2001). Present human studies do not support a relationship between running and OA unless abnormal joint conformation is present (Millis and Levine 1997). It appears that moderate exercise reduces the chance of OA either directly or through reduction of obesity (a major risk factor for OA in people (Felson et al. 1992)), or improved muscle strength and tone helping to stabilise joints (Millis and Levine 1997). Alterations in gait, joint alignment and ligamentous stability through injury may contribute to the development of OA. Changes in articular cartilage do occur with aging, however these changes are not identical to those occurring in OA and should not be confused (Millis and Levine 1997). Aging of equine articular cartilage appears to follow the same patterns as in humans (Platt et al. 1998)

Prolonged inactivity followed by physical activity may be detrimental to articular cartilage. Immobilisation of equine limbs by casting of a limb for 7 weeks resulted in loss of bone mineral density which, with the rehabilitation programme employed, resulted in remodelling and increased BMD associated with increased evidence of OA. These results suggested a negative effect of exercise during remobilisation of a limb subjected to reduced weight bearing for 7 weeks (van Harreveld et al. 2002b). This may be related to changes in articular cartilage composition within this time and inferior quality of articular cartilage to withstand resumption of normal weight bearing and a superimposed exercise programme.

The challenge exists to develop non-invasive means of distinguishing exercise-induced modelling and remodelling from pathologic change in vivo.

1.2.13.4 Chondrocyte viability

Viability is defined as 'the ability to live or exist particularly in a specific environment' (Pearsall 1998). The regulation of chondrocyte viability is important to maintain matrix stability (Horton et al. 1998). The loss of chondrocyte viability may play a significant role in the progression of OA (Levin et al. 2001; D'Lima et al. 2001b). Changes in matrix hydration alter passive chondrocyte volume, and if not compensated for by intra-cellular regulatory mechanisms could lead to altered cell volume, viability and matrix metabolism (Bush and Hall 2001b). Dead cells in mature articular cartilage are unlikely to be replaced, therefore the loss of even a small number of cells may have harmful consequences for the articular cartilage (Guilak et al. 1995).

a) Apoptosis and necrosis

Apoptosis is described as a mechanism whereby an organism removes cells that are not functioning efficiently or are not serving a function at a particular developmental stage (Horton et al. 1998), and differs from cellular necrosis (Table 1.1). Apoptosis in-vivo occurs in the hypertrophic zone of growth plates, but until recently there was no evidence to suggest it occurred in adult articular cartilage.

Apoptosis has now been suggested to play a role in maintenance and remodelling of mature articular cartilage. The reported apoptotic rate for normal and OA cartilage varies. In two comparative studies Heraud et al (Heraud et al. 2000) found an apoptotic rate in normal human articular cartilage of 2-5% and OA cartilage of 18-21%. Apoptotic rate has also been reported to be from 0.1% to 11% in normal articular cartilage (Sandell and Aigner 2001), and from 22 to 51% in patients with OA (Blanco et al. 1998; Hashimoto et al. 1998a; Hashimoto et al. 1998b; Kim et al. 2000). The higher rate of apoptosis found in some studies may be an overestimation as with rates of 5-11% it is difficult to comprehend that normal articular cartilage could maintain adequate matrix biosynthesis (Sandell and Aigner 2001). A rate of 0.1% would have minimal, if any, impact on articular cartilage matrix and may indicate a limited effect of apoptosis in the aetiopathogenesis of OA. Apoptotic rate increases with age (Adams and Horton 1998).

As apoptotic cells are not effectively removed from the matrix, the products of chondrocyte death may contribute to pathologic articular cartilage degeneration. Adams et al (1998) found the majority of apoptotic cells in young animals to be in the calcified cartilage zone of articular cartilage, which suggests a role of the SCB in the programmed death of these chondrocytes (Adams and Horton 1998). By contrast D'Lima (D'Lima et al. 2001b) reports few or no apoptotic chondrocytes in the deep zones, with most apoptotic cells in the superficial and intermediate zones. Heraud et al (2000) also found apoptotic chondrocytes to be mainly in the intermediate zone and superficial zone (when it was present) (Heraud et al. 2000; Tew et al. 2001).

Apoptosis has been shown to be induced by mechanical injury to chondrocytes as may occur during weight bearing or physical activity (Clements et al. 2001; D'Lima et al. 2001a). Another form of physiological chondrocyte death that does not involve apoptosis has been described with 'paralysed' chondrocytes identified as exhibiting signs not typical of classical apoptosis or necrosis. This mechanism is thought to be a mode of chondrocyte death that may prevent negative effects on neighbouring cells and does not require phagocytosis to remove by-products

(Roach and Clarke 2000). OA chondrocytes may be induced to undergo hypertrophic differentiation by external factors. The expression of the hypertrophic phenotype may induce chondrocyte apoptosis as occurs in growth plate cartilage, and result in loss of articular cartilage (Gibson et al. 1997).

Table 1.1: Comparison of the main features characteristic of apoptosis and necrosis.

Table adapted from Journal of Bone and Joint Surgery (Br) (Roach and Clarke 2000)

	Apoptosis	Necrosis
Cytoplasmic digestion?	No digestion, except by phagocytosis in other cells	Initial swelling and bursting of cytoplasm + organelles, vacuolation
Timing of nuclear and cytoplasmic events	Chromatin condensation precedes or is coincident with cell shrinkage	Nuclear condensation (pyknosis) + partial collapse of cell
'Blebbing' v 'budding' *	Budding into apoptotic bodies	Extensive blebbing
Pattern of chromatin condensation	Large, round or crescent-shaped masses at perimeter of rounded nucleus	Small patches throughout nucleus, nucleus usually rounded and condensed
Release of proteolytic enzymes?	No, lysosomes remain intact within apoptotic bodies	Yes, uncontrolled release of lysosomal enzymes
Function	Elimination of individual cells without inflammation	Unavoidable death of cells, inflammation

* The terms 'budding' and 'blebbing' have been used interchangeably by many authors. Roach and Clarke preferred to draw a distinction between the pseudopodia of apoptotic cells, described as 'budding', and the much smaller, blister-like, vacuolated structures for which the term 'blebbing' was reserved.

b) Response of chondrocytes in OA

Contrary to dogma, articular cartilage has some regenerative capacity. Chondrocytes can divide and increase synthetic rate, leading some authors to suggest a reversible nature to OA (Bland 1983). In OA the primary responses of the chondrocyte are to synthesise cartilage molecules and proteolytic enzymes. Chondrocytes react to degenerative changes in the articular cartilage in five different ways; 1) proliferation and cell death (apoptosis or necrosis); (2) changes in synthetic activity; 3) increased degradation; 4) phenotypic modulation of chondrocytes; 5) formation of osteophytes (Sandell and Aigner 2001).

The matrix molecules produced by chondrocytes during OA are type IIA and III procollagens which are typical of a more primitive phenotype (Sandell and Aigner 2001; Fukui et al. 2001). OA chondrocytes re-express the chondroproginator phenotype observed in foetal skeletal development. Reversion to a foetal-like phenotype occurs in the middle to deep chondrocytes with activated chondrocytes expressing type III collagen, procollagen and type X collagen (Aigner et al. 1993; Girkontaite et al. 1996).

Normal articular chondrocytes essentially have no proliferative activity, however OA chondrocytes demonstrate proliferation and clustering which are characteristic features of OA (Mankin et al. 1971; Hulth et al. 1972; Rothwell and Bentley 1973). Proliferation occurs with characteristic cell clusters, or clones, near the surface of the articular cartilage. Clusters of degenerate and viable articular cartilage cells have been reported to lie in areas showing advanced articular cartilage fibrillation (Mitchell et al. 1992). In humans the presence of clones consisting of large numbers of chondrocytes indicates that cell division can occur in adult OA cartilage (Oegema et al. 1997). Two types of clones were found in human OA cartilage, one involved in matrix synthesis and the other in degradation. The destructive clones lay closest to the joint surface and the synthetic clones deeper in the unaffected articular cartilage, giving weight to theory of OA beginning at the joint surface and progressing to deeper layers (Mitchell et al. 1992).

1.2.14 Viability staining and confocal microscopy

Cell viability implies that the cell has functional metabolic processes and an intact cell membrane to exist in its peri-cellular matrix. Assessment of chondrocyte viability using fluorescent stains is achieved by utilising the mechanism of entry of the stain into the cell. Determining the viability of ex vivo articular cartilage should give an indication of the physiological state of the tissue at the time of sampling. The selective uptake and staining of two fluorescent dyes is used to identify viable (live) and non-viable (dead) cells. The combination of Calcein AM and Propidium iodide is commonly referred to as a 'live/dead' stain.

Simultaneous vital staining by Calcein AM and propidium iodide has been used for viability determination in monolayer culture, but has not found wide application with thick tissue sections. Viability staining has been used widely to assess chondrocyte viability of ovine (Radcliffe 2001), bovine (Clements et al. 2001; Lucchinetti et al. 2002) and canine (Mainil-Varlet et al. 2001; Patricelli et al. 2001) articular cartilage. Viability stains and CLSM have

been used to study cellular and subcellular morphology, volume and surface area during mechanotransduction (Guilak 1994; Guilak et al. 1999; Lee et al. 2000) and the effect of impact loading on chondrocytes.

1.2.14.1 Calcein AM

Calcein AM is a fluorescent molecule which labels the cell cytosol and is widely used to detect membrane/cell integrity. The green fluorescent protein Calcein enters the cell as the weakly fluorescent and freely permeable ester (Calcein-AM) and is then cleaved by intracellular esterases to calcein which can then not exit the cell via intact cell membranes (Hibbs 2000). Green fluorescence is an indicator of viable cells that have esterase activity and an intact cell membrane that retains the esterase products (Figure 1.11). It was designed as a way to assay live and dead cells but has also been used to monitor cell volume changes during chemical hypoxia and to visualise the organisation of keratocytes in the stroma of intact living cornea. Chondrocyte volume and morphology changes in response to mechanical load and changes in osmolality and hydration can be monitored in situ (Bush and Hall 2001a) and in isolated chondrocytes (Hall and Bush 2001; Bush and Hall 2001b).

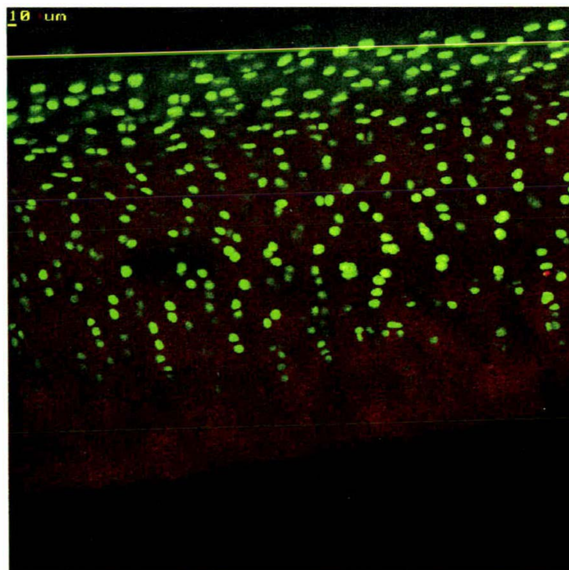


Figure 1.11: Image of articular cartilage (articular surface at the top of the image) with predominantly viable chondrocytes that have retained the fluorescent stain calcein. (16x objective).

1.2.14.2 Propidium Iodide

Propidium iodide is a high affinity, red, fluorescent nucleic acid dye that is able to pass through only damaged membranes of dead cells. The stain binds to nucleic acid and in doing so increases its fluorescent activity. Propidium iodide gives good fluorescence in the red channel (Figure 1.12) and has been widely used as a fluorescent stain (Tas and Westerneng 1981; Jones and Kniss 1987; Poulin et al. 1994; Suzuki et al. 1997). It allows simultaneous detection of DNA with other cell viability stains such as calcein (Wan et al. 1994). The excitation and emission wavelengths for both calcein and PI are relatively distinct, with the only limited cross-over providing two almost exclusive channels of fluorescence.

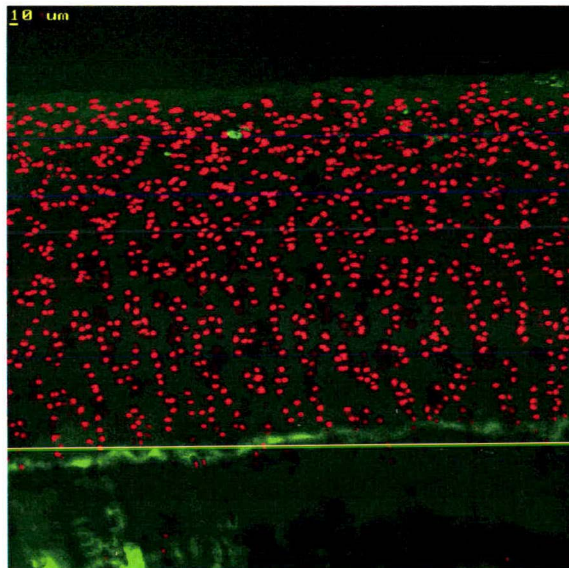


Figure 1.12: Image of articular cartilage, after heating to 80° for 30 minutes (articular surface at the top of the image), demonstrating dead chondrocytes. All chondrocytes have stained with propidium iodide (16x objective).

1.2.14.3 Confocal Microscopy

Confocal laser scanning microscopy (CLSM) was developed in the 1980's and has become a useful tool in biomedical research (Yuehuei H.An 1999). CLSM has been used to study a number of mammalian tissues including chondrocytes, cardiac myocytes, pancreatic, renal collecting duct, lung and sperm cells (Blatter 1999). It has allowed detection of fluorescence signals with high spatial and temporal resolution at variable cell depths (Blatter 1999) and has a wide variety of applications in orthopaedic research. It can be used to obtain thin optical sections of tissues that are relatively free of interfering autofluorescence and that do not strongly

scatter or absorb either the excitation or emission light (Paddock 1999). One approach to understanding aspects of cell volume and regulation involves the removal of the cells from the matrix and manipulation in culture (Raat et al. 1996). Measurements or assessment are also often required from cells within intact tissue that are operating in their correct physiological context. These include the effects of cell to cell interaction and the mechanical, ionic and physiological effects of the ECM. In situ approaches maintain tissue integrity and spatial organisation of cells within the ECM (Paddock 1999).

Fluorescence is the most important optical readout mode in biological confocal microscopy. It is much more sensitive and specific than absorbance or reflectance. Fluorescent stains that can be tagged onto biological macromolecules are used.

The main advantage of confocal microscopy is that it can provide not only 3-dimensional observations of an object, but also quantitative data such as thickness, area, volume and number of cellular structures. The optical sectioning capabilities of the Confocal CLSM eliminate the need for very thin tissue sections. CLSM exposes each fluorescent molecule to brief but intense bursts of excitation as the laser beam scans the specimen at a particular focal depth. Laser light through the objective excites the dye in the specimen, reflected light passes through the objective and through the out pinhole to an electronic detector. The pinhole is set at an optimum setting for the objective used such that any light above and below the plane of focus is rejected allowing only light from the specific plane of focus to pass through to hit the detector. The pinhole gives the CLSM its unique optical sectioning ability by omitting out of focus light from the focused image. This gives a sharp image undistorted by cellular structures above or below the plane of focus. A major advantage over conventional fluorescent microscopy is that the image is much sharper.

1.2.15 Computed tomography

Computed tomography (CT) has increasingly been used over the last 25 years to non-invasively quantify bone mineral density (BMD) in human patients with osteoarthritis (Markel et al. 1991). Computed tomography has been shown to provide superior imaging to radiography of the anatomical structure of bone, and to a lesser extent soft tissue, in the horse (Van Harreveld et al. 2002a). A rotating x-ray tube within a gantry emits radiation which passes through the patient and then detected opposite the beam. The amount of radiation attenuated by the tissue is calculated for each slice. Slice thickness is determined by the thickness of the collimated x-ray beam (Kraft and Gavin 2001). Each detector generates a CT number (Houndsfield unit (HU))

that is indicative of the radiation detected after attenuation by the tissue, expressed relative to the attenuation of water. In general, density of water is zero HU and air –1,000 HU, fat HU, cortical bone > +1,000 HU and cancellous bone approximately 700 HU. An average CT value of water is determined for each scan to adjust the systemic error of the machine (Ciarelli et al. 1991). The number is assigned a grey scale value which determines the shade of each pixel in the display image (Kraft and Gavin 2001). CT images represent a matrix of pixels, each of which represents a three dimensional block of tissue (voxel) from the body slice. The colour of the pixels represents the average linear attenuation co-efficient of the tissues in a particular voxel. A series of 2-dimensional images are generated, which can be reconstructed in other planes or rendered 3-dimensional to assess density patterns or location of lesions (Tucker and Sande 2001)

Computed Tomography (CT) has been used for examining bone structure, bone destruction and new bone formation during fracture healing and bone lengthening in animal models. Peripheral quantitative CT (pQCT) is capable of analysing bone structure and is believed to be more sensitive than dual-energy X ray absorptiometry (DEXA) (Henrotin et al. 2000). The development of QCT has resulted in images with high 3D resolution, which may be used for 3D reconstruction of cancellous bone (Kaab et al. 2000; Malinin and Ouellette 2000). QCT can be applied to in vivo or on excised bone specimens (Ciarelli et al. 1991). Mechanical values and densities of bone can be predicted by CT values (Ciarelli et al. 1991; Ferretti et al. 1996; Augat et al. 1997).

In the distal extremities, CT has been used to diagnose joint related problems which may be radiographically silent. The most notable benefit of CT is that it can detect a change in bone mineral density of 0.5% compared to the 30% change in bone mineral density necessary to be detected by conventional radiography (Kraft and Gavin 2001). Peripheral QCT is best for assessing density and subchondral sclerosis with no superimposition of over-lying structures (Hathcock and Stickle 1993), but small lesions such as enthisophtyes and osteophytes can be missed due to volume averaging. Bone mineral density (BMD) is expressed in grams per cubic centimetre (g/cm^3) in three dimensional analyses (Lepage et al. 2001). Computed tomography can be best interpreted along with high detail radiographs for thorough clinical diagnosis. In specific sites, remodelling changes in CT can be indicative of pathologic fractures and impending fracture (Tucker and Sande 2001). CT is a valuable tool for studies on the effects of conditioning regimens on equine bone.

1.3 Summary

The importance of chondro-osseous disease is evidenced by the launching of the current decade as "the bone and joint decade" in human osteoarthritis circles (Henrotin et al. 2000; Lohmander 2000). Considerable medical and veterinary research attention is now focused on OA. However, much remains to be done to further elucidate the sequence of events in OA across all species to improve welfare and reduce the economic implications of this insidious disease process. There is continued debate as to the relative involvement of articular cartilage and subchondral bone in the initiation of OA. Explanation of the predominant initiator in the cycle of OA will impact the development of diagnostic and therapeutic regimens for preventing or retarding its onset and progression.

The effect of exercise on developing articular cartilage and subchondral bone has been investigated in humans, laboratory and domestic animals, and more recently in the horse. The reported positive effects of gentle exercise and free paddock exercise on developing musculoskeletal tissues of the young horse leads to the proposition of controlled exercise regimens superimposed on naturally occurring paddock exercise, and their possible effect on articular cartilage and subchondral bone. Of particular interest are the effects of early exercise on chondrocyte viability and subchondral bone sclerosis. There are only limited data on the effects of early exercise on the joints commonly affected by osteochondral disease in the horse.

The cyclic intrinsic trauma of galloping leading to traumatic or exercise induced OA is the single most common cause of joint disease in athletic horses. The metacarpophalangeal (fetlock) joint, a high load, high motion joint, is commonly affected by OA. The distal third metacarpal bone has been shown to model and remodel in response to exercise. The palmar aspect of the condylar surface shows an increase in bone mineral density in a repeatable pattern, but the dorsodistal aspect does not.

The knowledge so far assembled leads to the metacarpophalangeal joint as the most suitable joint to study osteoarthritis and the effects of exercise on articular cartilage and subchondral bone. It is important to determine the relationship, if there is one present, of the articular cartilage and subchondral bone changes at the earliest stages of chondro-osseous disease. One way to do this is to assess young animals subjected to less than peak strains to study early articular cartilage and SCB changes that occur before end-stage disease.

Hence this study focuses on the relationship of articular cartilage integrity as assessed by vital staining, and subchondral bone status assessed by pQCT, in MCP/MTP joints of horses either

subjected to a known exercise regimen, compared to those of control animals exercising spontaneously at pasture only.

It was hypothesised that articular cartilage viability is influenced by exercise and loading within the MCP joint. Specifically we hypothesised that exercise and increased loading would lead to decreased chondrocyte viability which in turn is related to increased subchondral bone sclerosis. The results of this study may be used to further expand the knowledge on the initiating factors involved in very early OA and the effect of clinically applicable exercise programmes on the viability of developing equine articular cartilage and density of the adjacent subchondral bone.

1.4 Hypotheses

That in a given site (or sites) of the joint,

Hypothesis 1

That there is a statistically significant difference in the total number of chondrocytes between exercised and control horses.

Hypothesis 2

That the percentage of viable chondrocytes in exercised horses is significantly less than in control horses.

Hypothesis 3

That the percentage of viable chondrocytes, in the palmar and medial regions of distal Mc3 condyles, is significantly less than the dorsal and lateral regions.

Hypothesis 4

That the percentage of dead chondrocytes adjacent to areas of very high density subchondral (>0.76g/cm^3) bone is significantly greater than in areas with lower density subchondral bone (0.47-0.76g/cm^3).

Hypothesis 5

That the percentage of dead chondrocytes is significantly greater in areas close to the subchondral calcified tissues (Zone B) than in areas close to the articular cartilage surface (Zone A).

1.4.1 Objectives of this study

The purposes of this study were to:

1. Uphold or reject the hypotheses stated by conducting a study on articular cartilage and subchondral bone of 12 young Thoroughbred horses (6 exercised and 6 control).

2. Analyse and interpret the findings.

3. Discuss the findings in the context of previous and future study aimed at preventing or reducing the impact of OA in equine athletes.

Protocols are presented for:

1. Examining chondrocyte viability using calcein and propidium iodide staining and confocal laser scanning microscopy.

2. Assessing subchondral bone mineral density using quantitative computed tomography determination of bone mineral density of the distal third metacarpal bone epiphysis.

CHAPTER 2 MATERIALS AND METHODS

2.1 Definitions

BMD - Very high	Greater than 0.76 g/cm^3
BMD - High	0.67-0.76 g/cm^3
BMD - Moderate	0.57-0.66 g/cm^3
BMD - Low	0.47-0.56 g/cm^3
BMD - Very low	less than 0.47 g/cm^3
Confocal image	The digital output of the confocal microscope.
Explant	The 3mm diameter portion of articular cartilage harvested from each site.
Field	One of three areas of the fresh sample examined by confocal microscopy and used to create the images for chondrocyte counting.
Region	The dorsal or palmar, medial or lateral aspect of the metacarpo/metatarso-phalangeal (MCP/MTP) joint.
Sample	The articular cartilage plug was bisected into two samples, a fresh sample and a fixed sample.
Site	One of four locations of sampling within the MCP joint, or two locations in MTP joint. Site is defined by a combination of the regions, for example, the dorsopalmar site.
Slice	One of the optical sections created during a confocal depth scan in a plane perpendicular to the articular surface.
Stack	Serial contiguous optical slices constitute a stack which is 10µm thick, 625µm x 625µm and 512 x512 pixels.
Viable	Uptake of calcein-AM and resulting green fluorescence indicating chondrocyte viability.

2.2 Animals

Thirty-one Thoroughbred foals born in the 2000 breeding season at Flock House Thoroughbred Research Stud, Bulls, New Zealand, were matched for sire and sex, and allotted into treatment (exercise) and control (non-exercised) groups for a research project investigating the effect of early exercise on the musculoskeletal system. From birth the horses were managed together at pasture in paddocks of approximately two hectares. As foals they were weaned at 4 months of age. Their diet was predominantly ryegrass/clover pasture-based, with supplementary meadow hay during winter.

From 10 days to 18 months of age the treatment group underwent a programme of incremental controlled exercise superimposed on their normal paddock activity. The horses were encouraged from 10 days post partum to trot, canter and gallop on a purpose-built track. The velocity of the horses was regulated by the use of two four-wheeled farm bikes equipped with mobile barriers. The horses were exercised in both a clockwise and counter-clockwise direction with equal frequency. The track surface was turf covered with a thin layer of sand to improve the consistency of the surface and prevent slipping during wet conditions.

The exercise regimen consisted of exercise five days a week over 1020 metres. Speed and distance were altered during the musculoskeletal development of the horses to increase the intensity of the exercise. The number of lay-up periods for the exercised horses were also recorded. The exercise load was increased twice during the study. Initially the foals exercised at a constant base speed of 4.20m/s, which was increased to 5.56m/s in February 2001 when the foals were 4 months of age. At 6 months of age the base speed was increased to 6.66m/s and a 250m sprint at 12m/s was introduced after the first 500m at base speed.

The behaviour patterns of the exercised and control group were monitored. Observations included the duration of rest, grazing and play periods during the day. All horses were observed by the stud manager daily for clinical abnormalities. Condition scores and weighing were carried out twice weekly. Full clinical examinations were conducted monthly by experienced equine clinicians. Any veterinary treatment was recorded in individual foal records. Twelve of the horses (6 control and 6 exercised, both groups consisting of three geldings[1] and three fillies) were humanely euthanased at 18 months (±30 days) of age. Samples from both metacarpophalangeal (MCP) joints and the left metatarsophalangeal (MTP) joints were used in this study.

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[1] The geldings were castrated as yearlings.

2.3 Joint Dissection

Within 30 minutes of euthanasia, the skin and flexor tendons were removed from the limb. The MCP or MTP joint was opened via the palmar pouch of the joint capsule using aseptic technique. The palmar pouch was dissected to the collateral ligament which was cut, and the dorsal portion of the joint capsule transected. The joint was partially opened to expose the distal third metacarpal bone (Mc3) and proximal first phalanx (P1). The relationship between proximal P1, proximal sesamoid bones (PSB) and distal Mc3 was maintained by the contra-lateral collateral ligament and palmar joint capsule.

The joint cartilage was examined under bright light and articular cartilage abnormality was recorded. Following sample collection the joint tissues were re-approximated, the dorsal joint capsule sutured closed, wrapped in cling film and frozen at –20°C for computerised tomography at a later date. An identical protocol was used when dissecting the left metatarsophalangeal joint.

2.4 Articular Cartilage Sample Collection and Preparation

Articular cartilage explants were taken from four sites (dorsolateral, dorsomedial, palmarolateral and palmaromedial) of the distal condyles of the left and right Mc3, and from two sites (palmarolateral and palmaromedial) of the left distal Mt3.

The dorsal and palmar sites were consistently identified by using an aluminium triangle with 25° and 35° angles placed at the centre of the collateral ligament insertion (the centre of rotation of distal Mc3) (Figure 2.1A). A notional line was drawn at the level of 35° from the centre of rotation of the condyle across the palmar, and 25° from the centre of rotation of the condyle across the dorsal, aspects of Mc3. The sample sites were identified on the palmar and dorsal lines midway between the articular margin (medial and lateral) and the junction of the condylar surfaces and the median sagittal ridge (Figure 2.1B, Figure 2.1D). A sharp 3mm leather punch was manually advanced into the articular cartilage at the predetermined sites (Figure 2.1C). A scalpel blade was used to notch the very edge of the distal aspect of the explant to orientate the articular cartilage for later bisection in the frontal plane. The sample was removed with gentle use of a chisel and hammer. Approximately 0.5 mm of subchondral calcified tissue was attached to the explant. Fine atraumatic forceps were used to handle the explants.

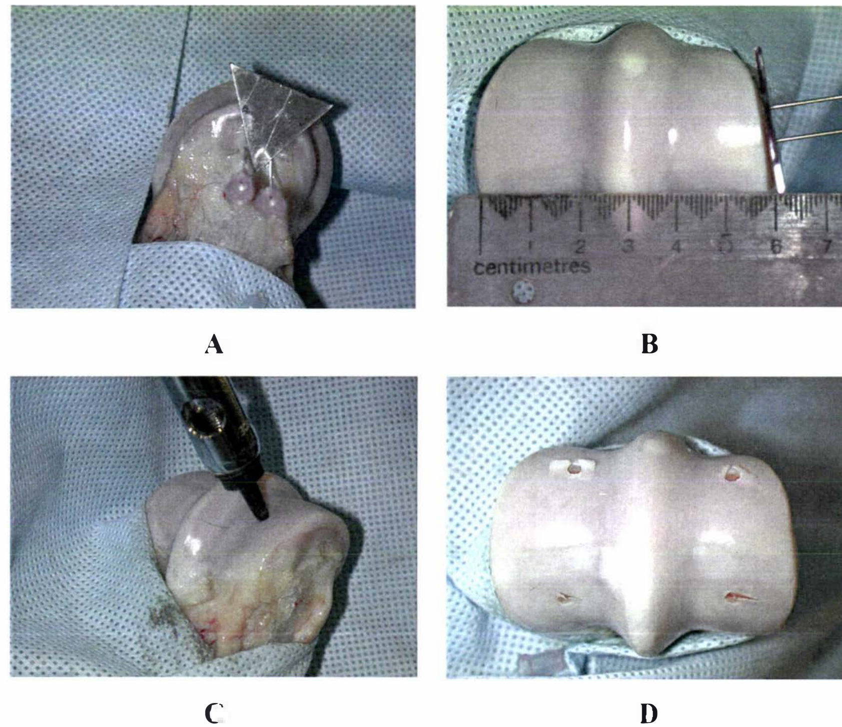


Figure 2.1: Images of the articular cartilage explant location and sampling technique.

Image A: An aluminium triangle with the apex placed at the centre of attachment of the collateral ligament was used to identify a line 35° palmar and 25° dorsal for the centre of rotation of the distal metacarpal/metatarsal bone. Image B: Lateral and medial sites were located along the line, midway between the articular margin and median sagittal ridge. Image C: The 3mm leather punch was manually advanced into the articular cartilage. Image D: The four sample sites of the distal metacarpal/metatarsal bone after explant removal.

The explants were immediately identified by horse number, limb and site, and immediately placed in sterile Dulbecco's modified eagle media, DMEM (Life Technologies Ltd, Auckland, New Zealand), and kept at 37°C in DMEM for 4-20 hours until staining with fluorescent viability dyes for 30 minutes prior to confocal laser scanning microscopy (CLSM). Careful tissue handling, sharp instrumentation and the shortest possible interval between harvest and microscopy were employed to reduce artefactual damage to chondrocytes.

Pilot work had indicated that chondrocyte survival was not adversely affected within 72 hours of euthanasia in unopened equine MCP joints stored at 4°C. It had been observed (Bush, personal communication), that bovine articular cartilage explants in culture media at 37°C for up to five days had no appreciable chondrocyte death, but that there was significant chondrocyte death, particularly in the superficial layer, of articular cartilage in bovine MCP joints stored unopened at 4°C for more than a few days. Based on this information all explants were placed

into DMEM within three hours of euthanasia and stained and examined within 24 (± 6) hours of collection.

2.5 Confocal Microscopy

2.5.1 Viability staining

Immediately prior to staining and microscopy, the explants were bisected in the frontal plane from the articular surface to SCB using a fresh razorblade. One half of the explant (Figure 2.2) (fresh sample) was placed in 1ml of DMEM with 5µl of 1mMol stock solution (Appendix 1) of propidium iodide (PI)(Sigma, Aldrich Pty, Sydney, Australia) and 5 µl of 1mMol stock solution of calcein-AM (Molecular Probes Inc, Life Technologies Ltd Auckland, New Zealand). The samples were incubated in a water bath at 37°C for 30 minutes. Washing the samples prior to microscopy was not necessary as both stains exhibit weak fluorescence prior to intracellular and nuclear binding. To preserve fluorescence the PI and calcein were protected from light during storage and staining. The other half of the explant (fixed sample) was placed in 10% buffered formalin for later histological examination (Figure 2.2).

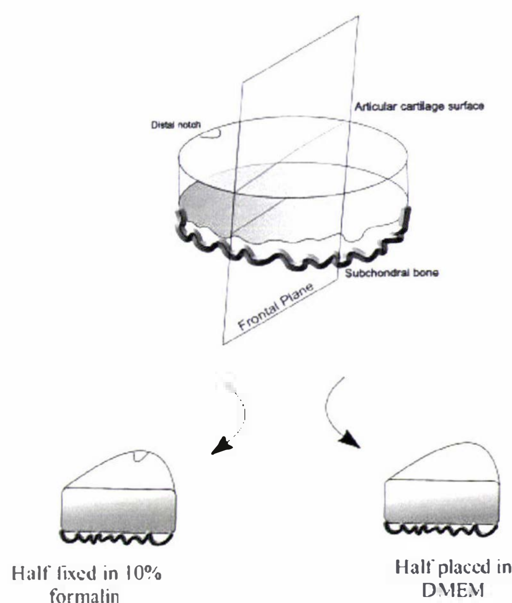


Figure 2.2: Diagram of the bisection plane of the articular cartilage explants.

One of the halves was placed in DMEM for viability staining and confocal microscopy, the other fixed for histological preparation.

2.5.2 Microscopy

The fresh sample was mounted in a conforming support material (Blutack™) with the cut surface uppermost. Glycerol and a coverslip were applied (Figure 2.3).

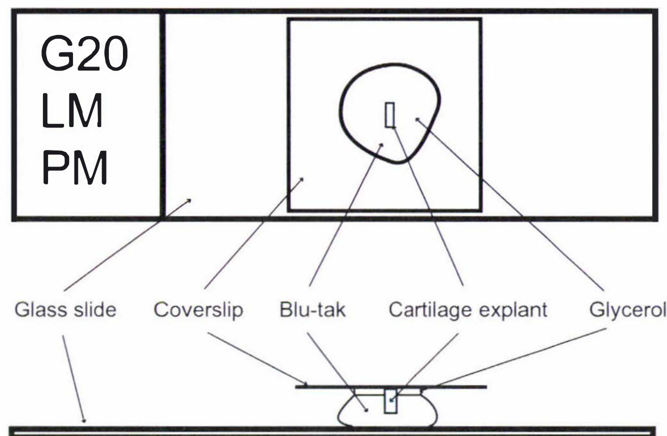


Figure 2.3: Diagram of a mounted confocal microscopy slide.

The fresh articular cartilage sample is placed in conforming support material with a cover slip overlain. The slide is identified with the horse number and site.

An upright Leica DMRBE confocal laser scanning confocal microscope with a Leica TCS 4D scanning head and Kr/Ar mixed gas laser was used (Leica, Heidelberg, Germany) to collect image data. The optical plane scanned was parallel to the cut surface from the articular cartilage to SCB.

Samples were initially analysed in ultraviolet epi-fluorescence mode using the 10x objective and an eye piece graticule to measure the depth of articular cartilage from articular surface to tideline at three sites along the approximately 3mm length of the surface.

The median emission frequencies of calcein and PI are approximately 495nm and 635nm respectively (Figure 2.4). Dual scan filter arrangements were used to view calcein and PI simultaneously. Dual excitation was at 488nm and 568nm, using a dichroic mirror (488/568nm) excitation wavelengths were reflected away, allowing the emission wavelengths through. The emission wavelengths were then split into two channels, the calcein emission (channel one) and the PI (channel two). Light was picked up by the relevant detectors and split into the two separate channels using a beam-splitter filter at 580nm, which reflected wavelengths lower than

580nm into channel one and letting the longer wavelengths through into channel two. In channel one, a further band-pass filter was used to capture only wavelengths between 515-545nm for calcein and in the second channel a long-pass filter let all wavelengths above 590nm through to the detector which allowed the capture of the PI emissions.

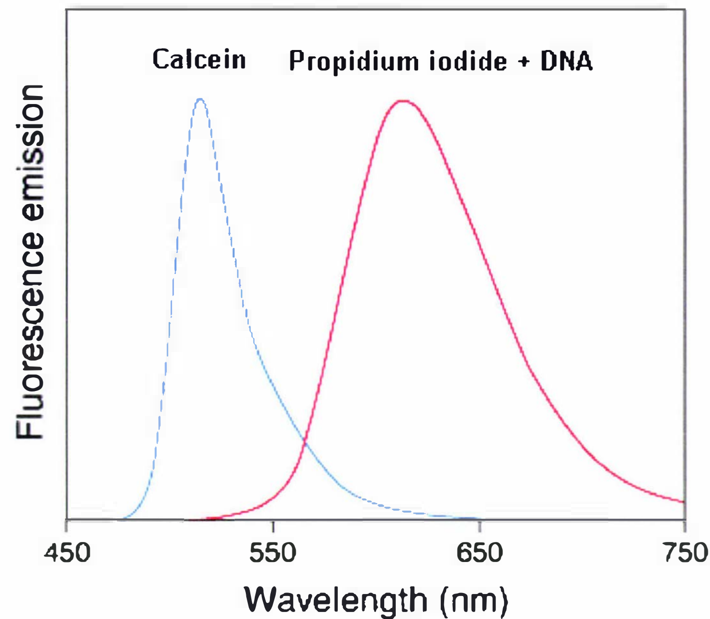


Figure 2.4: Fluorescence emission profiles for calcein and for propidium iodide bound to DNA. From Molecular Probes product information 2003

Different combinations of microscope settings were trialled during pilot experiments to determine the optimal setting for examining the articular cartilage for counting of chondrocytes. A 16x oil immersion objective, pinhole aperture of 23, standard scan speed with a line average setting of 8, was found to be optimal. Laser power was set to a sufficient level to excite the calcein and PI molecules to fluoresce. The colour of the fluorescence distinguished between chondrocyte cytoplasm encased in an intact cell membrane (bright green fluorescence), and exposed nucleic acid due to disruption of the chondrocyte membrane (red fluorescence), from the background extra-cellular matrix (black). Background fluorescence due to the collagen component of the ECM was negligible.

To obtain confocal images representing articular cartilage sections of 10µm depth a continuous scan was performed to locate and orientate the cut surface and obtain an on-screen image from the articular surface to SCB. From the cut surface the articular cartilage was scanned to a depth of at least 20µm to avoid cut edge artefact. A depth scan was defined by setting more and less deep bottom positions 10µm apart. A series scan was carried out to obtain a stack of five optical sections through this 10µm depth. Microscope settings were constant for each field.

Laser power varied between fields and samples to obtain best contrast between chondrocytes and the ECM.

Three fields of interest were identified in each sample and the confocal image stacks obtained (Figure 2.5). The fields were separated by approximately 250µm and were at least 250µm from the trephined cut edge of the sample. Based on pilot work, when a new razor was used to cut fresh articular cartilage, cell death at the cut surface was no greater than 20µm. Confocal image stacks were created by combining five serial optical sections from each field within the sample. Scanware software (Leica, Heidelberg, Germany) produced images of 625µm x 625µm x10µm, (512 pixels x 512 pixels) for counting chondrocytes. Each stack was saved as an individual .tif file (512 pixels x 512 pixels) and the stack identified by a Leica .inf information file. The .inf file contained details of the microscope and laser settings used to collect the stack.

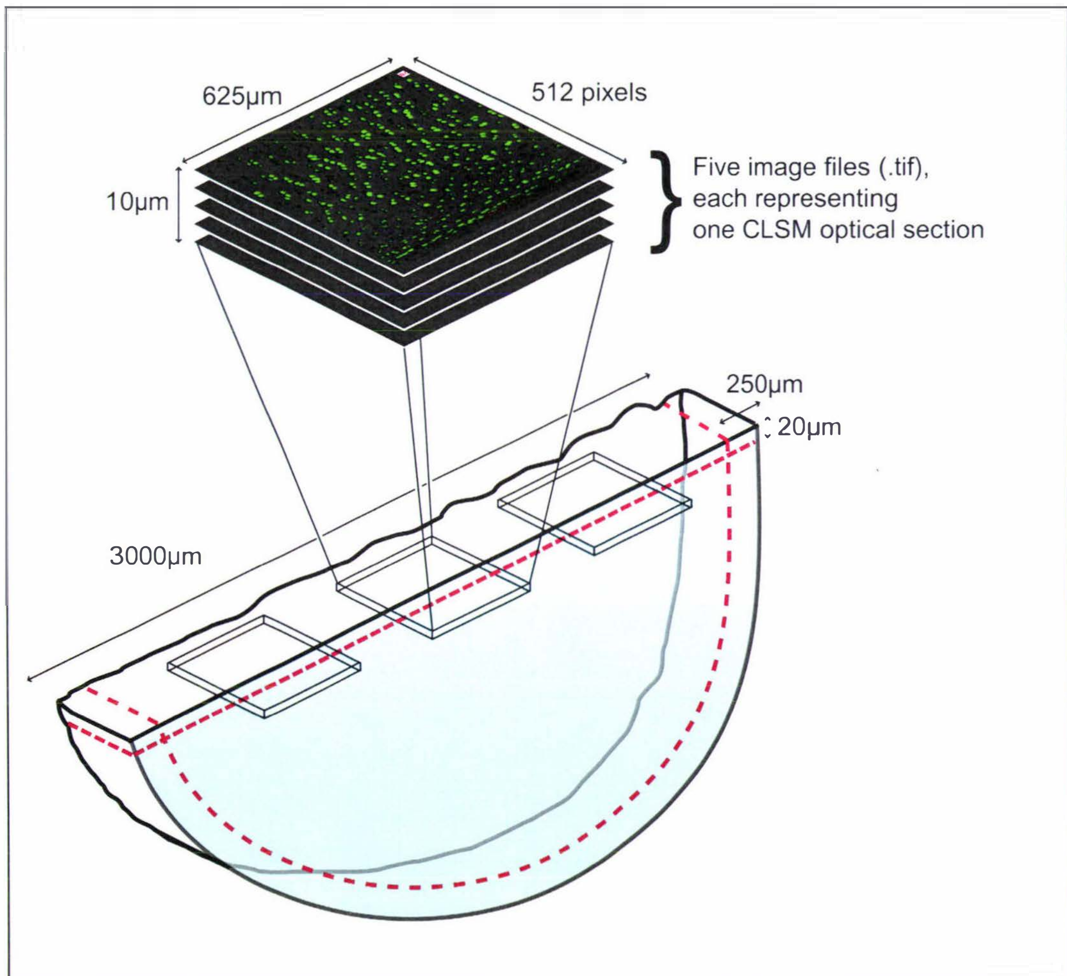


Figure 2.5: Diagram of a fresh articular cartilage sample.

The cut surface is uppermost and the articular surface shaded blue. Three fields of interest are shown in the sample demonstrating the 'stack' of optical sections obtained during CLSM that combined to produce a 10µm x 625µm x 625µm image used for counting the chondrocytes.

The stack depth of 10µm was found to provide the best differentiation between chondrocytes for counting. When articular cartilage depth exceeded 625µm two scans were necessary to display the complete image. The first scan was of articular surface to a depth of 625µm and a second scan taken of the area between the bottom of the first and the tideline. Chondrocytes in the non-calcified cartilage were consistently observed up to the tideline. Chondrocytes in the calcified cartilage zone were inconsistently visualised, possibly due to variable SCB thickness and stain penetration into the calcified tissues in the allocated staining time.

2.6 Computed Tomography

Bone mineral density (BMD) of left and right distal Mc3 and left distal Mt3 was determined using quantitative computed tomography (CT) (Figure 2.6). Computed tomographic scans were made with a Picker Luminys spiral CT scanner (Phillips Medical, USA) at 1.5-mm slice intervals. All joints were scanned with a K_2HPO_4 density CT calibration phantom within the field of view. The CT scan files were imported to a PC desktop for image analysis using the CSU OsteoApp software. CT data points were converted from CT numbers (Houndsfield Units), which is a relative measure of density based upon water, to g/cm^3. The known density points were obtained from a conversion involving a K_2HPO_4 density phantom. The conversion was calculated with a formula published by (Genant et al. 1982):

$$C_b = (H_b\text{-}H_w/H_k\text{-}H_w)*C_k$$

Where C_b is the actual g/cm^3 density of the pixel, H_b is the Houndsfield unit of the pixel, H_w is the HU of water in the phantom, H_k is the HU of the K_2HPO_4 in the phantom, and C_k is the known K_2HPO_4 density of the phantom.

Once the scans were converted, the third metacarpal condyles were isolated as regions of interest determined by the density threshold of 700 HU. OsteoApp software was then used to build a three-dimensional model containing only those data points included in the regions of interest. The 3D model was manipulated to display only the distal articular surface of the third metacarpal condyle and surface density measurements calculated to a depth of 2mm. The BMD was measured 2mm proximal to the articular cartilage subchondral bone plate junction, immediately underlying the site of articular cartilage explant removal. The regions of interest (ROI) were initially 3mm x 3mm x 2mm and later increased to 6mm x 6mm x 2mm. BMD was

expressed as g/cm3 for each ROI. Palmar and dorsal volumised slice views were used and BMD for the medial and lateral sites determined.

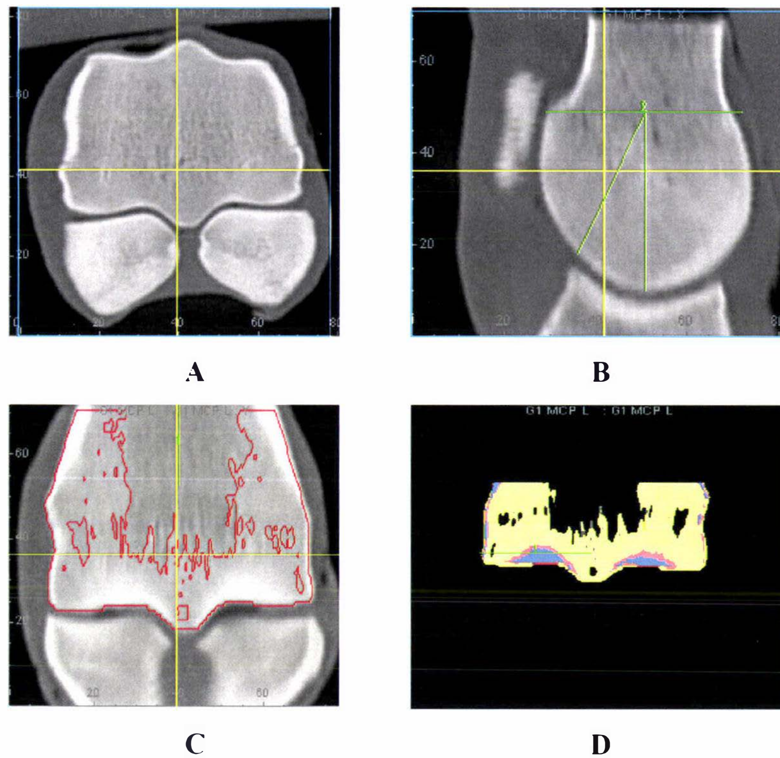


Figure 2.6: Images of pQCT data collection.

Image A: CT data is first aligned parallel to the sagittal ridge. Image B: A line of intersection is drawn for the pivot point, a 35° angle is made toward the palmar surface and the slice positioned. Image C: The distal metacarpal/metatarsal bone ROI was isolated (750-2000 HU). Image D: Two mm thick palmar and dorsal transverse sections used to measure BMD under the articular cartilage sample sites.

2.7 Image Analysis

The counting of chondrocytes (viable and dead/compromised) was standardised by using image analysis software. Confocal images were counted using Image-Pro Plus (version 4.5 for Windows, Media Cybernetic, Silver Springs, MD, USA).

Segmentation, the process of dividing the image into objects of interest, was used in combination with thresholding to consistently separate the objects (chondrocytes) from the background (ECM). Thresholding was performed to correct for the variation in nuclear and

cellular brightness and the small amount of background fluorescence. Pixels that were above a predetermined intensity, when compared to the background, were designated as object, and those below the intensity were designated as background. The threshold value was set by using a semi-quantitative brightness scale to provide the most precise estimate of chondrocyte numbers and viability. Throughout the experiment constant thresholding criteria were applied. Twenty images were assessed for mean background pixel intensity and a subjective threshold chosen for each one to optimally identify chondrocytes. The background pixel intensity and threshold were plotted (Appendix 2). This provided a line of best fit that was used subsequently to determine threshold values for the images prior to counting. The effect of small differences in the pixel intensity of the background on the counting of the cells was minimised by this method.

Two filters were applied in a constant order. A medium filter, followed by an open filter, was used to separate adjacent and contacting chondrocytes before counting. If the automated processes did not resolve two co-joined chondrocytes then a watershed split and auto-split function was used if required. A manual split process was available if obviously touching cells were not separated by the prior functions.

The confocal image was divided into two zones for counting. Zone A consisted of the tangential and intermediate zones of articular cartilage, while Zone B was the deep zone. A horizontal line was drawn through the image at the junction between zones A and B as determined using the semi-quantitative criteria (Appendix 3).

2.8 Histological Scoring Systems

Histological sections were prepared from the fixed half of the articular cartilage explant. The staining protocols for Safranin O/Fast green (SOFG) and Haematoxylin and Eosin (H&E) are described in Appendix 4. The areas examined using confocal microscopy and the histological sections were within 1.5mm of each other.

2.8.1 Histomorphologic examination

Fixed and decalcified sections of articular cartilage, with attached SCT, were stained with Haematoxylin and Eosin (H&E) and examined blindly by a single examiner for degenerative and physical changes in the superficial, intermediate and deep articular cartilage zones.

Sections were evaluated for morphologic change using the semi-quantitative Modified Mankin Score (Foland et al. 1994; Frisbie et al. 1997; Kawcak et al. 1997; Frisbie et al. 1998). The modified Mankin scoring system used the following criteria for classification,

Articular cartilage fibrillation (scored 0-4)

0 no fibrillation
1 surface fibrillation
2 clefts into the tangential zone
3 clefts into the radiate zone
4 clefts into the calcified cartilage layer

Fibrillation is the state in which the articular surface no longer appears structurally intact when examined by light microscopy. Mild fibrillation is confined to the superficial layer of articular cartilage while more severe fibrillation extends into deeper layers.

- Chondrocyte necrosis (scored 0-3)
- Chondrone formation (scored 0-3)
- Focal cell loss (scored 0-3)

(0 no change, 1 mild, 2 moderate, 3 severe changes).

The total modified Mankin score was determined for each section with a maximum possible score of 13.

2.8.2 Histochemical examination

Fixed and decalcified sections were stained with safranin O and fast green (SOFG) and examined blindly by a single examiner for staining intensity of the superficial, intermediate and deep territorial and deep inter-territorial zones of the articular cartilage on a scale of 0-4 (Foland et al. 1994). The intensity of the stain uptake semi-quantitatively assessed the amount of proteoglycan in the articular cartilage.

The SOFG scoring system used the following criteria,

0 no stain uptake
1 25% of normal stain uptake
2 50% of normal stain uptake

3 75% of normal stain uptake

4 normal stain uptake

An overall SOFG score was determined for each section with a maximum possible score of 16.

2.9 Statistical Analysis

Data collected in Image-Pro Plus were collated and analysed in SPSS 10.1.3 (SPSS Inc; Chicago, IL, USA). One data point was missing for chondrocyte cell counting, histomorphometry, and histochemical analysis.

Variables in the data sets examined included horse, sex (gelding and filly), group (exercise and control), leg (fore and hind limb, left and right limb), site (palmar and dorsal, medial and lateral) and subchondral bone mineral density. Horse was coded as a random effect. All other variables were coded as fixed effects. For all analysis the significance level was set at $p<0.05$. The results were presented graphically using box plots, bar charts and frequency plots. In the boxplots, the top and bottom of the box represent the upper and lower quartiles enclosing the middle 50% of the data. The middle horizontal line indicates the median, and the 'T' shaped lines represent the 2.5^{th} percentile and the 97.5^{th} percentile, with outliers shown as open circles. Unless otherwise stated the data are presented as mean ± standard deviation.

Only the palmar sites were collected from the hindlimb. There was no significant difference between the palmar sites in the forelimbs and the hind limb for any of the variables, and data were thus pooled for analysis.

Before analysis, data were tested for normality. For normally distributed data the statistical tests performed were paired samples t-test and a univariate general linear model. If the univariate general linear model was significant, differences between variables were tested with least significant difference (LSD) post hoc tests. Differences between groups of non-normally distributed data were tested using the Mann-Whitney test. Where applicable a Monte-Carlo estimate was used to detect significant differences, as it was accurate for small sample sizes. Odds ratios were generated from cross tabulation of data in Excel. The relationship of the percentage of dead chondrocytes and underlying BMD was tested using linear regression models.

CHAPTER 3 RESULTS

3.1 Growth Rate and Work Load

There was no significant difference in growth rate between the control and exercised foals. The work load of the exercise group over the course of the study is displayed in Figure 3.1.

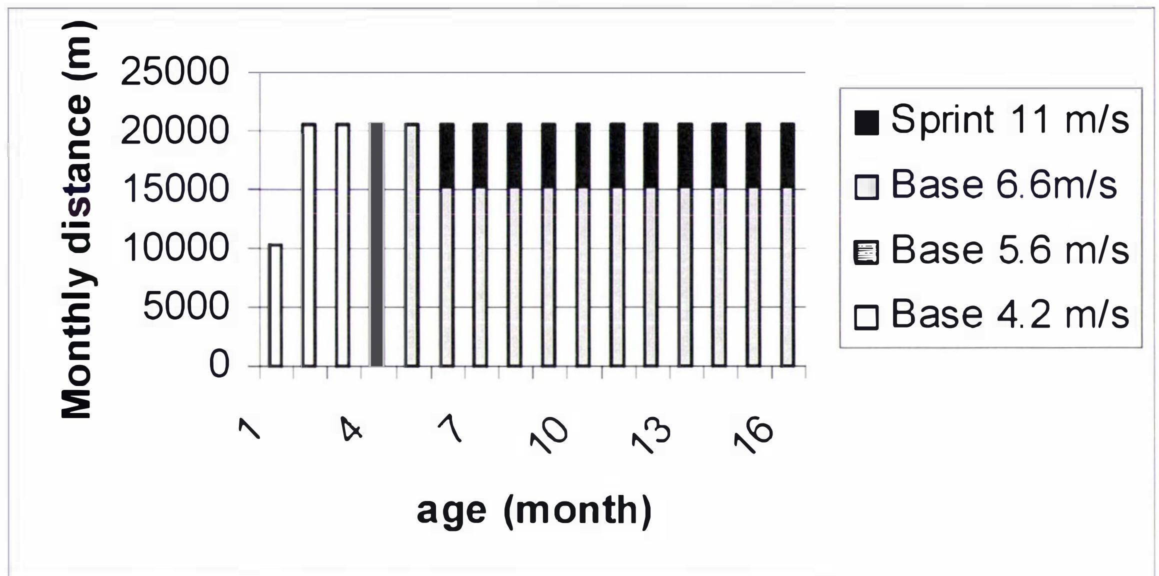


Figure 3.1: Workload of exercised group over study period.

Based on measurements of the animals' behaviour as foals at pasture, controlled exercise superimposed on normal paddock exercise had no significant effect on overall paddock activity. Exercise appeared to produce a temporal shift of the animals' daily routine. The control group grazed while the exercised group was exercising, and so control animals would rest earlier during the day than the exercised animals. The exercised group of foals were observed to graze for a short time immediately after exercise and then rest.

3.2 Medical History

The exercised group did not exercise for two weeks in June 2001 due to mild respiratory disease. Exercise was resumed once the animals were judged to be fit to exercise by the attending veterinarian. A small number of minor conditions (eg: subsolar abcessation, minor abrasion or laceration) required medical treatment and/or a short rest period during the study. The problems did not significantly affect the overall work load of the animals. In the six

months from November 2001 to April 2002, six of the horses (4 exercised, 2 controls) were examined by a veterinarian for problems most often due to injury sustained during exercise or at pasture (Table 3.1).

Table 3.1: Musculoskeletal conditions and injuries occurring within 24 weeks of euthanasia.

Horse No.	Treatment Group	Physical examination	Weeks prior to euthanasia
1	Exercised	Left hindlimb (LH) foot abscess.	24
		Reoccurrence of LH foot abscess	8
16	Exercised	Bilateral swelling of forelimb proximal sesamoid bone (PSB) area. Pain on flexion of right forelimb (RF) fetlock joint.	15
		Superficial shoulder laceration	14
20	Exercised	LH lameness – undetermined origin	16
		Accident on horse float during transport to slaughterhouse, multiple abrasions and contusions.	0
31	Exercised	Right forelimb (RF) foot abscess	24
		Superficial injury to left thorax	20
		Right hindlimb (RH) fetlock area skin abrasions and swelling.	10
		RH fetlock area swelling.	8
3	Control	Swollen RF proximal cannon bone.	20
		Bilateral swelling of forelimb PSB.	12
		Bilateral swelling of forelimb PSB.	8
17	Control	RF foot lameness	20
		RH stifle lameness	14
		RH hoof wall crack at toe	8

3.3 Gross Pathology

Gross pathology was evident in the articular cartilage of five proximal phalanges, two of the distal third metacarpi and one distal metatarsus (Table 3.2). The other MCP/MTP joints appeared grossly normal. Of these six horses with gross lesions to the articular cartilage of the metapodial bones five (83%) were from the control group.

Table 3.2: Gross pathology within metacarpo/tarsophalangeal joints at dissection.

Horse number	Group	Site	Lesion
13	Control	Right forelimb (RF) medial dorsoproximal eminence of the proximal phalanx (P_p)	Partial thickness articular cartilage erosion
17	Control	Left forelimb (LF) median sagittal ridge of the distal third metacarpal bone (Mc3)	Type 1 healed osteochondrosis (OCD) lesion*
24	Control	LF median sagittal groove of P_p	Mild partial thickness articular cartilage erosion
		LF median sagittal ridge Mc3	Mild partial thickness articular cartilage erosion
30	Control	LF medial dorsoproximal eminence of P_p	Mild partial thickness articular cartilage erosion
		RF medial dorsoproximal eminence of P_p	Mild erosion
33	Control	RF medial dorsoproximal eminence of P_p	Small loose chondroid fragment (Figure 3.2)
20**	Exercise	Left hindlimb (LH) proximal P_p and distal Mc3	Moderate-marked articular cartilage erosions and cartilaginous proliferation

* Type 1 osteochondrosis lesion defined as fragmentation of the articular cartilage surface in a typical OCD site (Hurtig and Pool 1996)

** Haemorrhage in multiple joints associated with ante-mortem trauma.

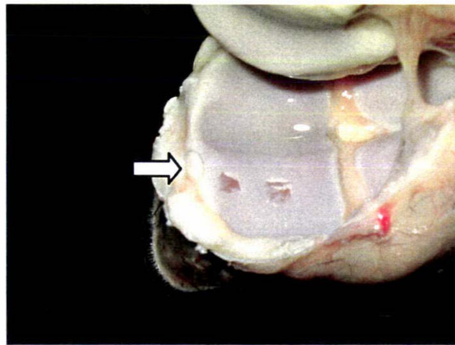


Figure 3.2: Image of the right MCP joint of horse No.33 with a dorsomedial P_p chondroid fragment (arrow).

3.4 Non-calcified Cartilage Thickness of Mc3

Irrespective of treatment group, there was a large variation in the non-calcified articular cartilage thickness between horses. The left hind fetlock joint of horse No. 20 showed gross evidence of severe osteoarthritis. Preliminary examination of the data indicated that values from the two sample sites in this joint were outliers for articular cartilage depth (> 1.0mm). For this reason data from the left hindlimb of No. 20 was removed from the data set before final pooled analysis.

The thickness of the non-calcified articular cartilage (from articular surface to tideline) was not significantly different between exercise and control horses for data pooled across all sites. However, there was a significant difference in the depth of the non-calcified articular cartilage thickness between dorsal and palmar regions when interaction with treatment group (exercised and control) was included in the analysis (Figure 3.3).

The non-calcified articular cartilage thickness of the palmar region of the exercised horses was significantly different between the dorsal region of exercised group (p=0.011) and control group (p=0.043) of horses. The difference between the palmar region of the exercised group and the palmar region of the control group just failed to reach significance (p = 0.060).

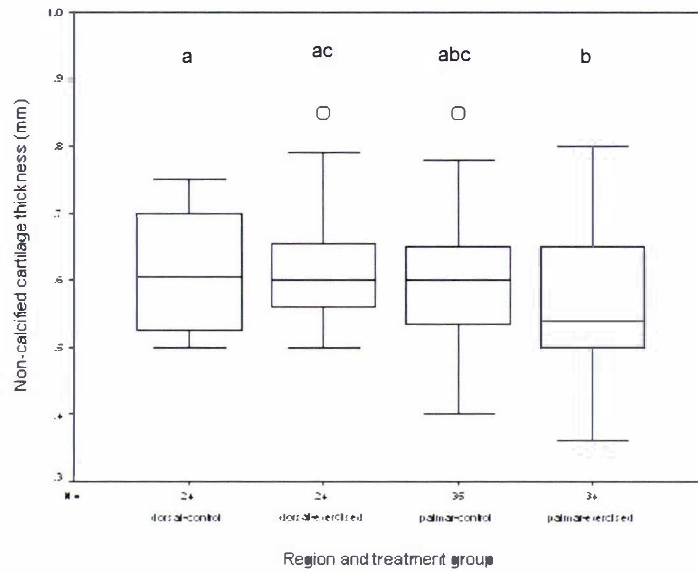


Figure 3.3: Box and whisker plot comparing the non-calcified articular cartilage thickness in control and exercised horses between dorsal and palmar regions.

Lower case letters (a,b,c) indicate those regions which are significantly different from each other.

3.5 Total Number of Chondrocytes

The total number of chondrocytes was counted for each field and then corrected for cartilage depth, providing a value per mm of cartilage thickness. Irrespective of treatment group there was a large inter-horse and intra-horse variation in the pooled total chondrocyte number (Figure 3.4). Inter-horse variation was not a statistically significant effect in any of the models tested.

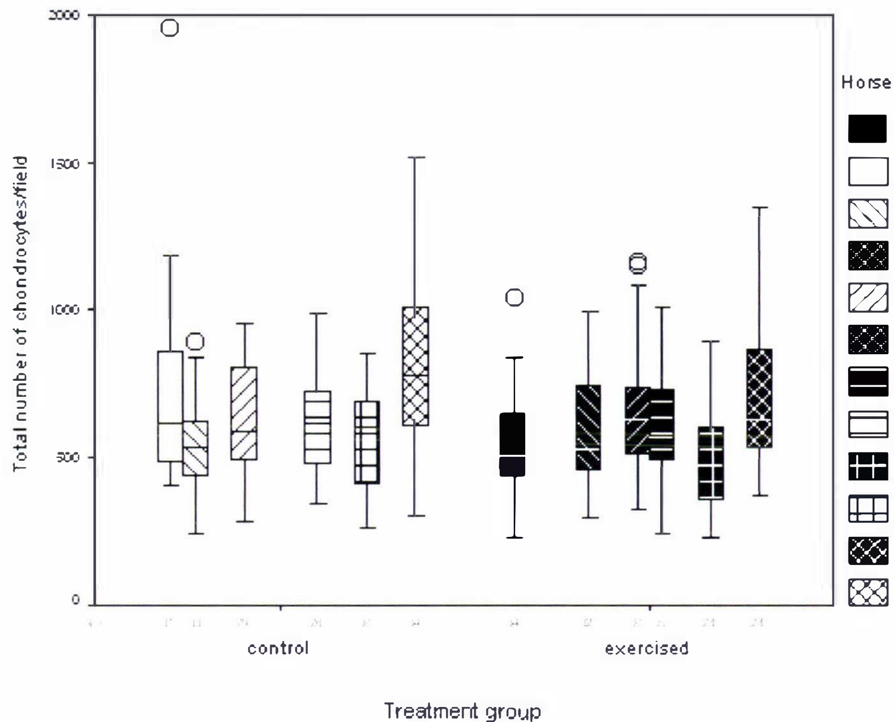


Figure 3.4: Box and whisker plot of pooled total number of chondrocytes per field for individual horses. At least three confocal microscopy images from 10 samples from the three joints of each horse were counted.

Forelimbs vs hindlimb

Only palmar region samples were obtained from the left hindlimb. To test if there was a significant difference between forelimbs and the hindlimb, pooled palmar forelimb and pooled hindlimb sites were analysed for significant differences.

The total number of chondrocytes for palmar sites in the left hindlimb was not significantly different from palmar sites of either forelimb. Therefore pooled forelimb and hindlimb data were used in the analysis of total chondrocyte number for pooled treatment group and region data.

Exercise vs control

The total number of chondrocytes pooled from all sites was not significantly different between treatment groups.

Palmar vs dorsal

The total number of chondrocytes was significantly different for pooled palmar and dorsal sites (Figure 3.5). The palmar region had a 13% greater mean total number of chondrocytes than the dorsal region (p=0.003). There was no interaction with treatment group.

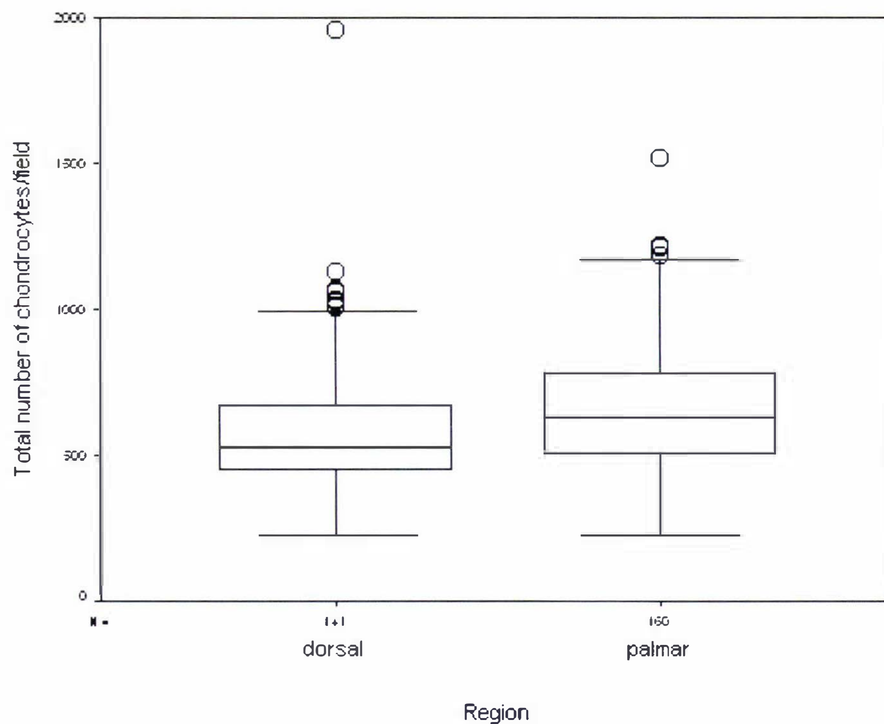


Figure 3.5: Box and whisker plot of the total number of chondrocytes for pooled dorsal and palmar sites.

Medial vs lateral

The total number of chondrocytes was not significantly different between lateral and medial sites. There was no interaction between treatment group and medial or lateral regions.

Left vs right limb

Mean total number of chondrocytes (pooled sites) in the left forelimb was 13% greater than in the right forelimb ($p=0.02$) (Figure 3.6).

There was no interaction with treatment group for total number of chondrocytes in left and right forelimbs.

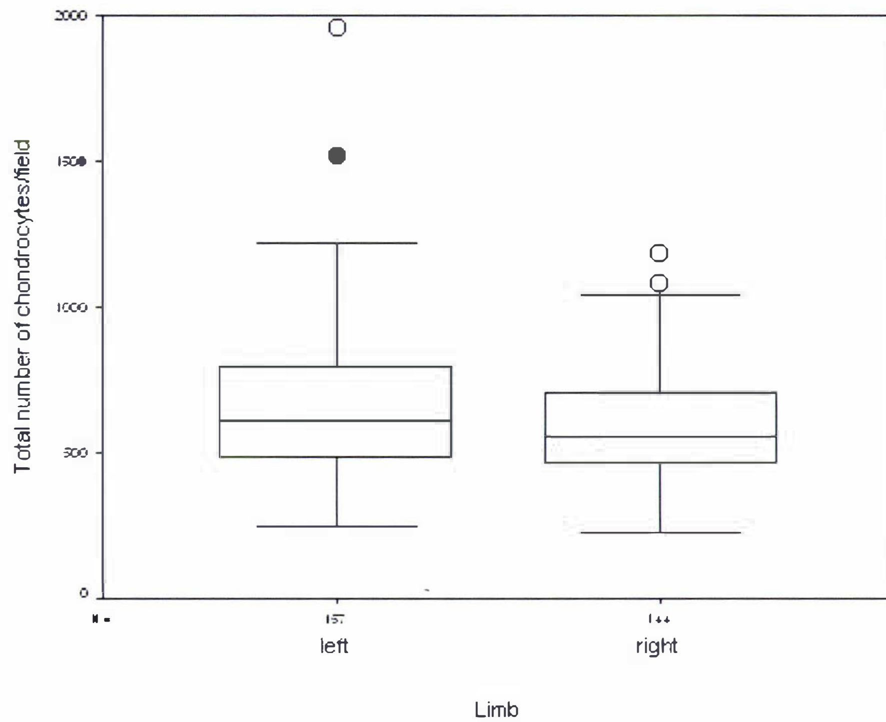


Figure 3.6: Box and whisker plot of the total number of chondrocytes for pooled left and right forelimb sites.

3.6 Percentage of Viable Chondrocytes

As was observed with the total number of chondrocytes, there was a large inter-horse and intra-horse variation in the percentage of viable chondrocytes across pooled sites.

Forelimbs vs hindlimb

The percentage of viable chondrocytes was not significantly different in left forelimb and left hindlimb palmar sites. The left hindlimb sites were included in analyses of the data set. An interaction with treatment group was identified for limb with the forelimb exercised sites having 5% greater mean percentage of viable chondrocytes than the hindlimb exercised, and 15% greater than the forelimb control (p=0.017) (Figure 3.7).

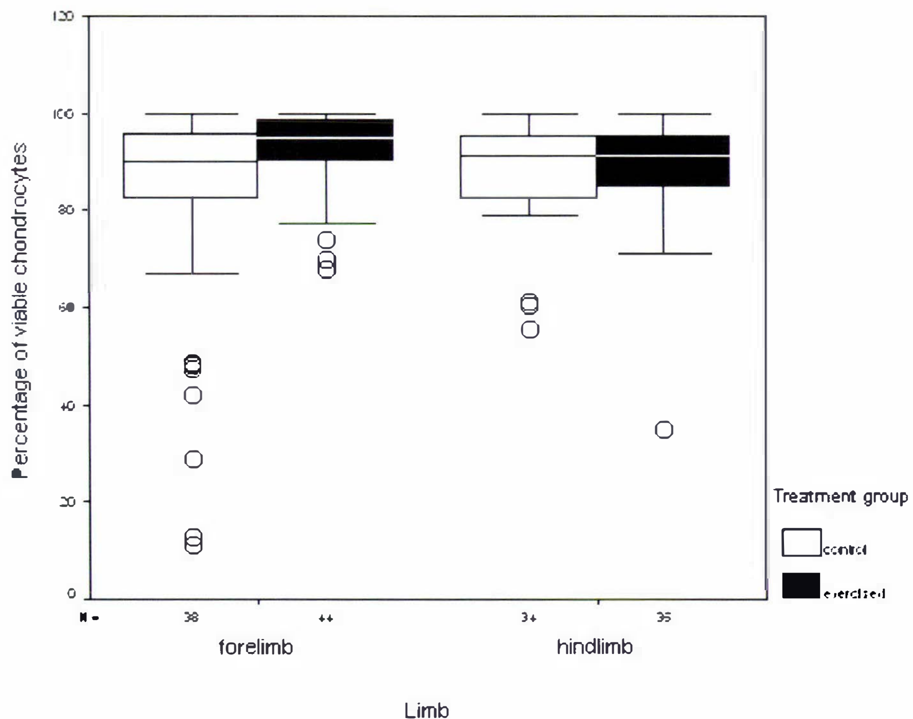


Figure 3.7: Box and whisker plot of the percentage of viable chondrocytes for forelimbs and hindlimb by treatment group.

Colts vs fillies

The mean percentage of viable chondrocytes from all sites was 5% greater in colts than fillies, which was a significant difference (p=0.034). There was a significant sex and treatment interaction (Figure 3.8). For all sites control colts had a mean percentage of viable chondrocytes 21% greater than control fillies (p= 0.001).

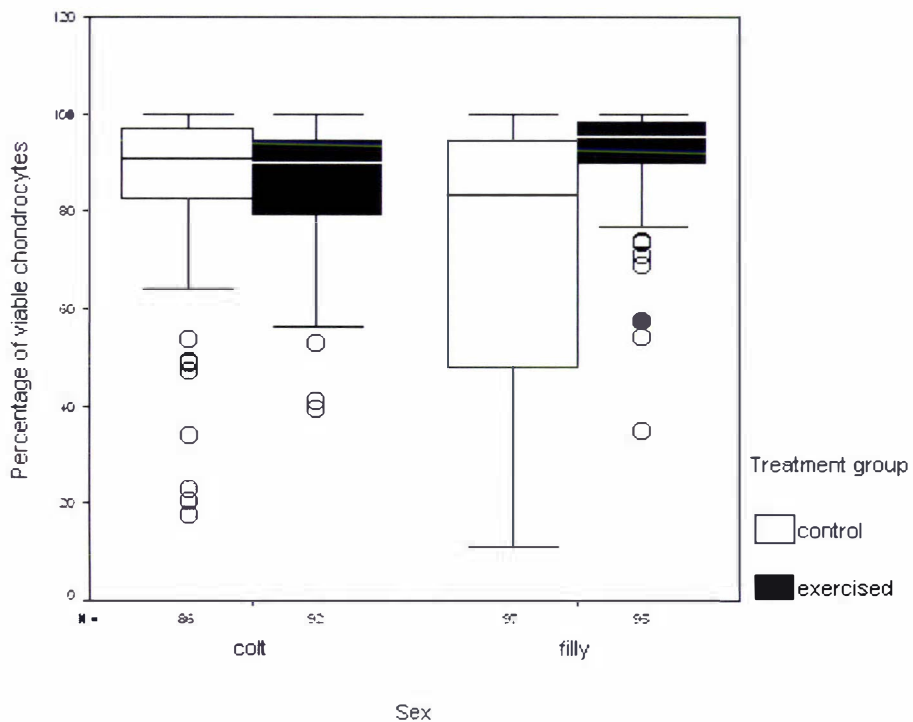


Figure 3.8: Box and whisker plot of the percentage of viable chondrocytes in colts and fillies by treatment group.

Exercise vs control

The mean percentage of viable chondrocytes (pooled sites), was 14% greater in the exercised group than the control group (p=0.001) (Figure 3.9).

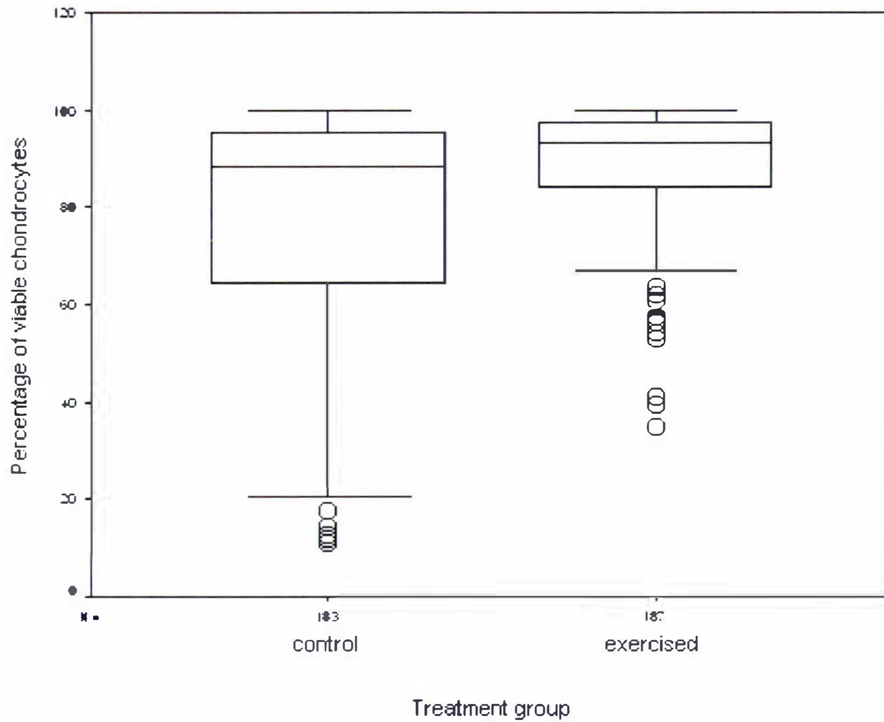


Figure 3.9: Box and whisker plot of the percentage of viable chondrocytes in exercised and control horses for data pooled across all sites.

The mean percentage of viable chondrocytes was 34% greater at dorsal sites of the exercised group than at control group sites (p=0.001) (Figure 3.10).

Variability in the percentage of viable chondrocytes was much less in the exercised group (both sites), and in palmar sites of both the exercised and control groups (Figure 3.10).

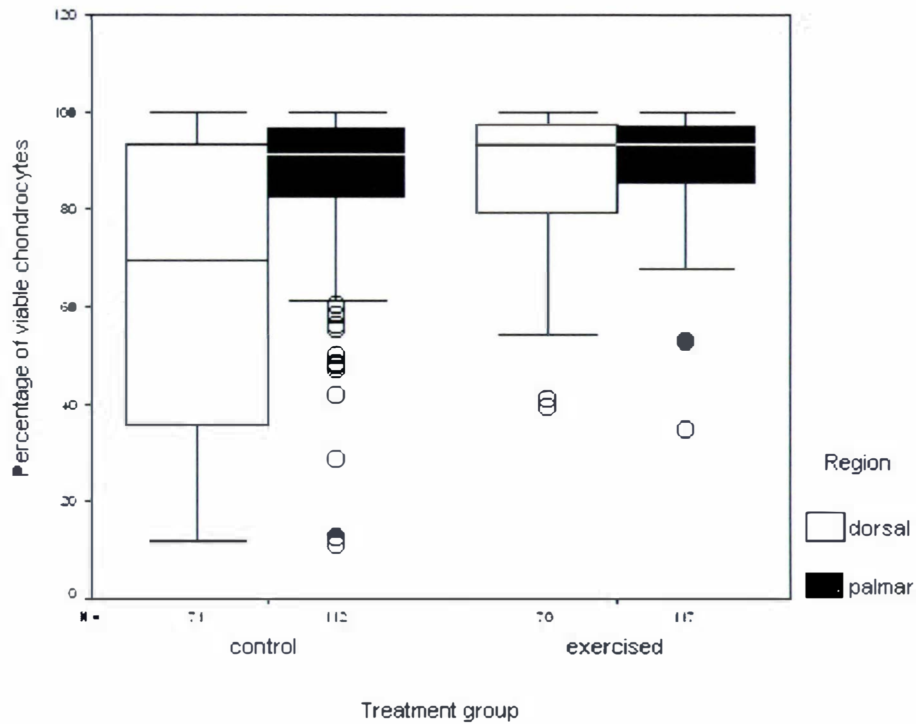


Figure 3.10: Box and whisker plot of the percentage of viable chondrocytes in exercised and control horses at dorsal and palmar sites.

Palmar vs dorsal

Irrespective of treatment group, the mean percentage of viable chondrocytes (pooled data) was 16% greater, and variability was much less, in palmar than dorsal sites (p=0.001) (Figure 3.11).

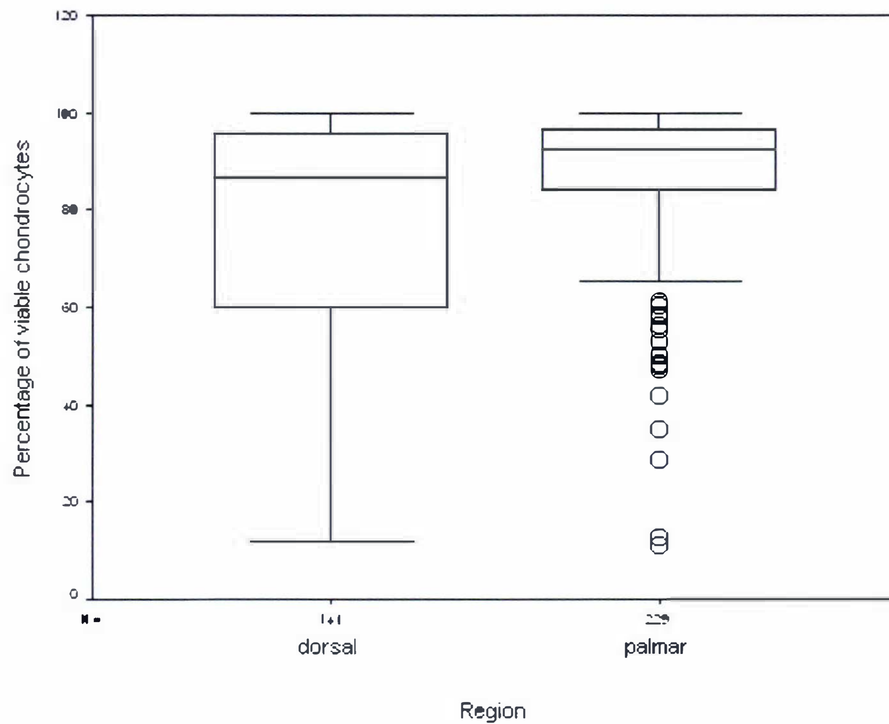


Figure 3.11: Box and whisker plot of the percentage of viable chondrocytes in the pooled dorsal and palmar sites.

There was a significant interaction with treatment group driven by the dorsal region (p=0.001) (Figure 3.12). The mean percentage of viable chondrocytes in palmar control sites was 33% greater than in dorsal control sites. The percentage of viable chondrocytes, when analysed by palmar or dorsal region, exhibited a similar pattern to that observed between exercised and control groups: There was less variation in percentage of viable chondrocytes in the palmar sites, than in the less loaded dorsal sites (Figure 3.12).

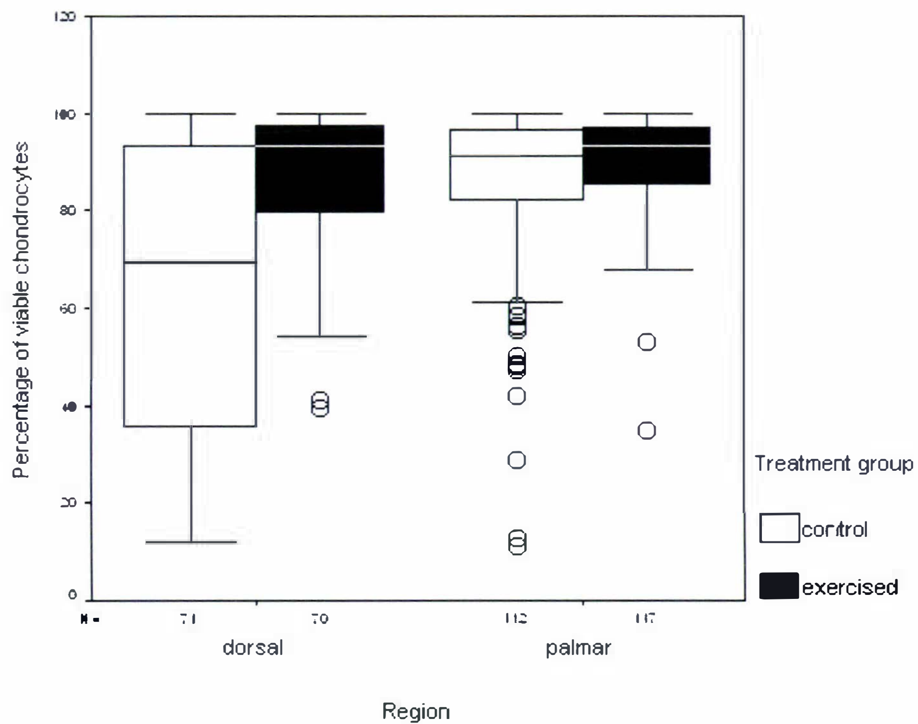


Figure 3.12: Box and whisker plot of the percentage of viable chondrocytes at dorsal and palmar sites by treatment group.

Medial vs lateral

The percentage of viable chondrocytes for pooled data was 6% greater in the medial compared to the lateral sites (p=0.012) (Figure 3.13), with no significant interaction with treatment group or region.

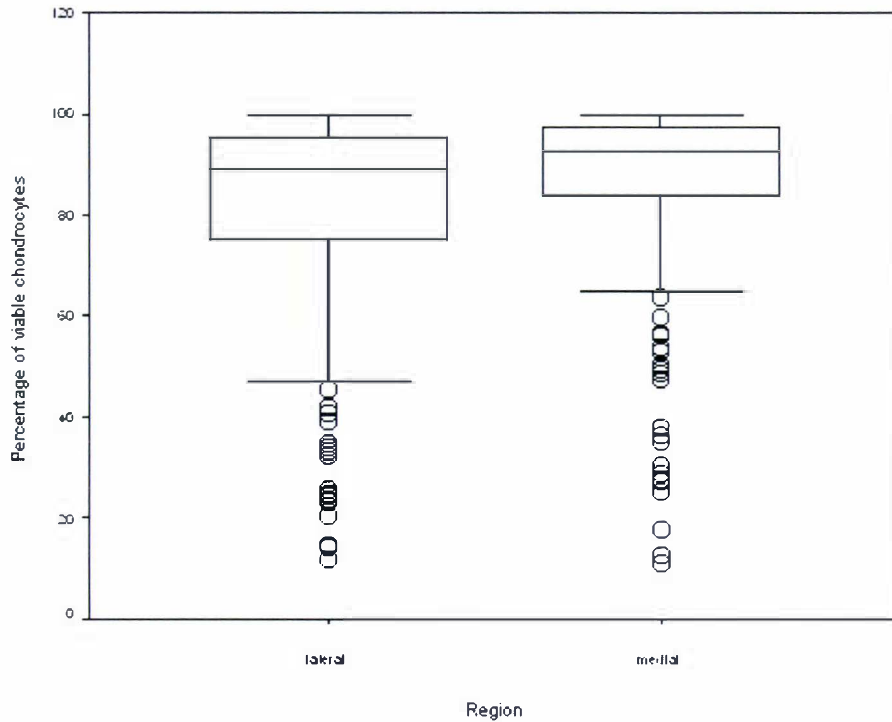


Figure 3.13: Box and whisker plot of percentage of viable cells at pooled lateral and medial sites.

3.7 Subchondral Bone Mineral Density

The percentage of voxels in five different bone mineral density (BMD) value ranges (described previously as very low (<0.47 g/cm^3) – very high (>0.76 g/cm^3)) within the regions of interest (ROI), is shown for treatment groups at dorsal (Figure 3.14) and palmar regions (Figure 3.15).

The BMD range is skewed to the right in the palmar regions with higher percentage of high density voxels in both the control and exercised groups. This skew is not obvious in the dorsal sites.

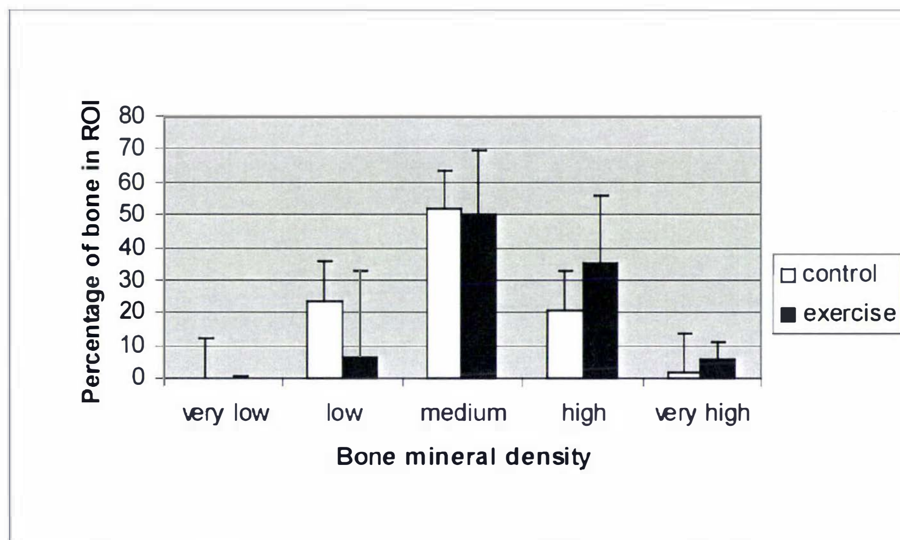


Figure 3.14: Bone mineral density ranges in the dorsal regions of control and exercised horses.

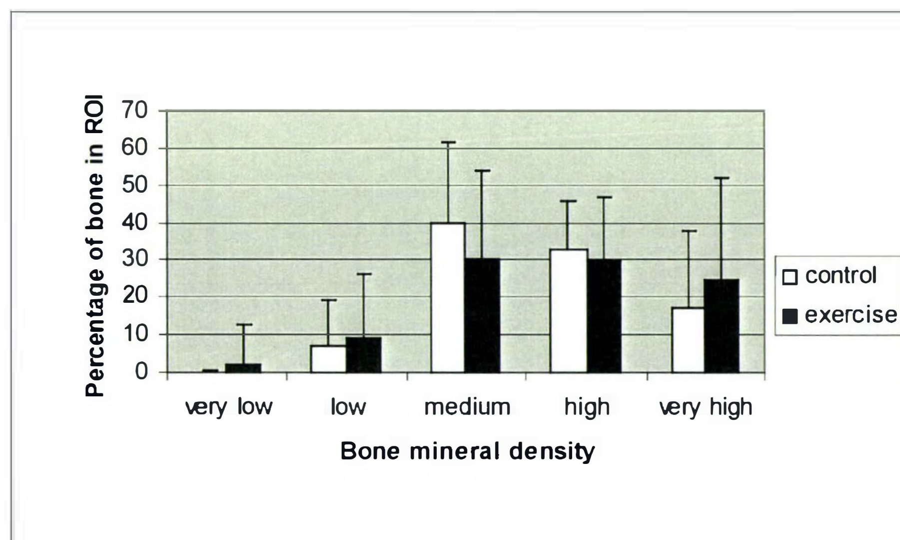


Figure 3.15: Bone mineral density ranges in the palmar regions of control and exercised horses.

The mean BMD (Figure 3.16) at the ROI was not significantly different between exercised and control groups.

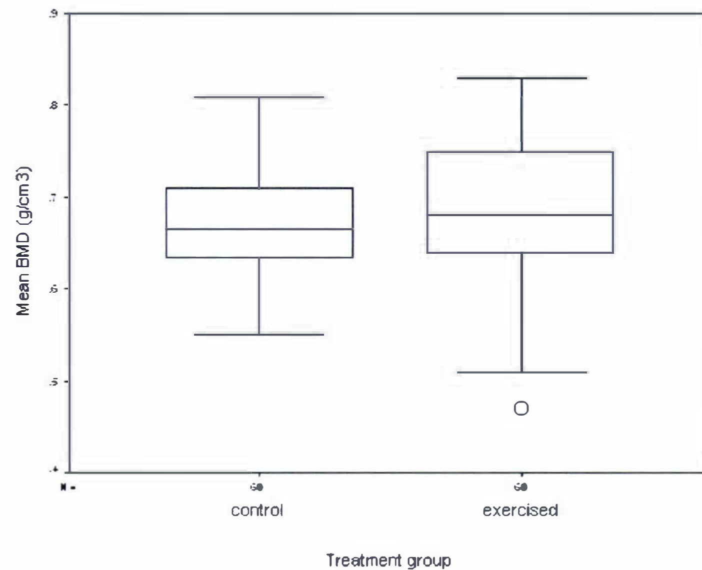


Figure 3.16: Box and whisker plot of the mean BMD for exercise and control groups pooled across all sites.

The mean percentage area of very high density bone (>0.76 g/cm^3) in the ROI was 70% greater in the exercised than the control group, but this difference was not significant (p=0.1)(Figure 3.17).

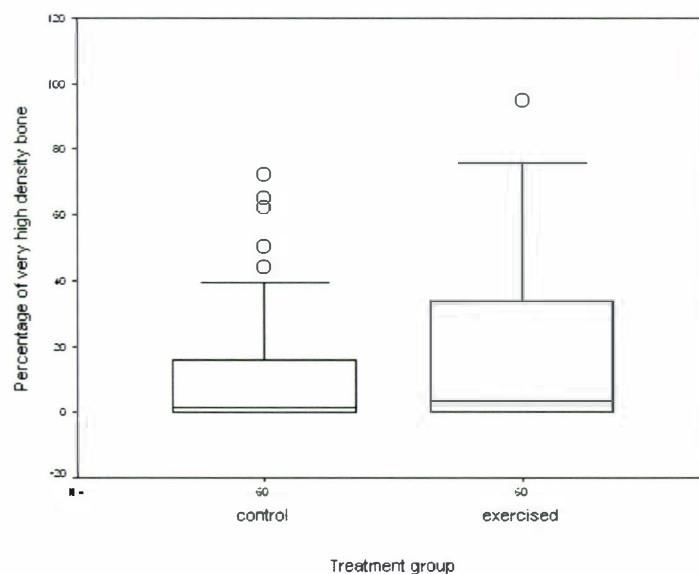


Figure 3.17: Box and whisker plot of the percentage of area of very high density bone (> 0.76g/cm^3) for exercised and control groups pooled across all sites.

When examined by region, the mean percentage of very high density bone was 5.6 times greater in palmar (20.8%) than dorsal sites (3.8%) (p=0.001) (Figure 3.18)

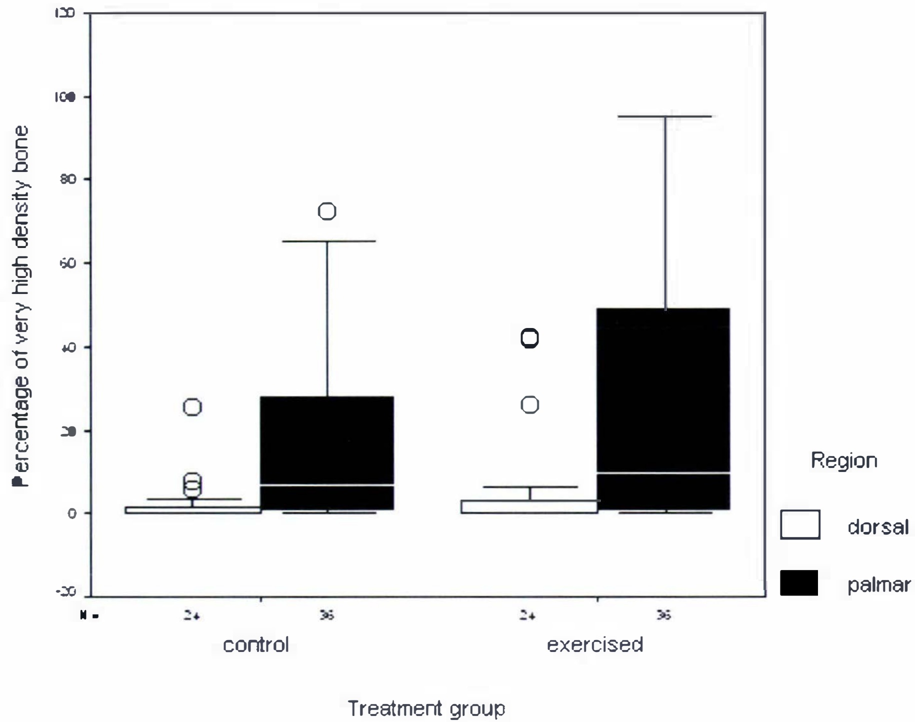


Figure 3.18: Box and whisker plot of the percentage area of very high density bone for exercise and control groups at palmar and dorsal regions.

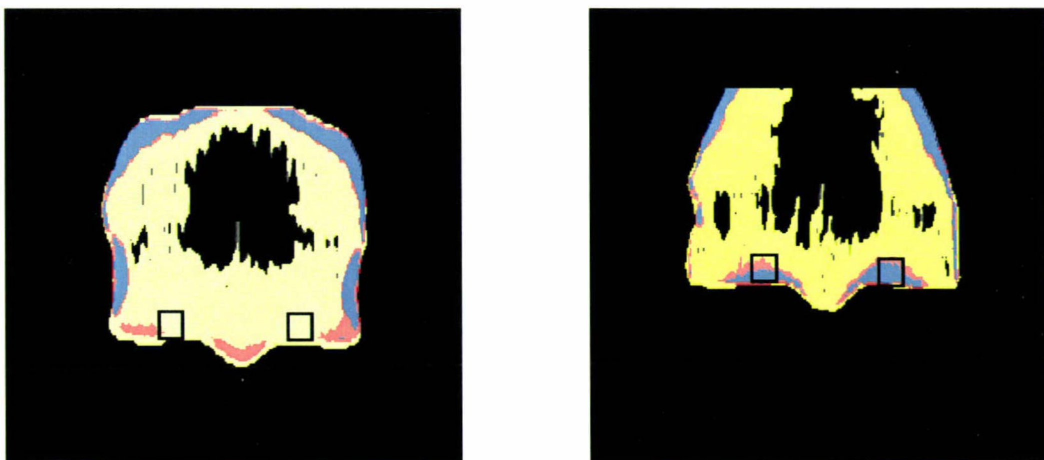


Figure 3.19: pQCT images of dorsal (left) and palmar (right) slices of distal Mc3.

The sites of the ROI are indicated as square boxes. The density of each voxel of the image is indicated by colour from yellow (low) to blue (high).

3.8 Percentage of Dead Chondrocytes and SCB Mineral Density

The relationship of % of dead chondrocytes and mean SCB density at ROI

There was no significant relationship of % of dead chondrocytes with mean BMD at the ROI across pooled exercised and control group data, $r^2 = 0.0034$ (Figure 3.20).

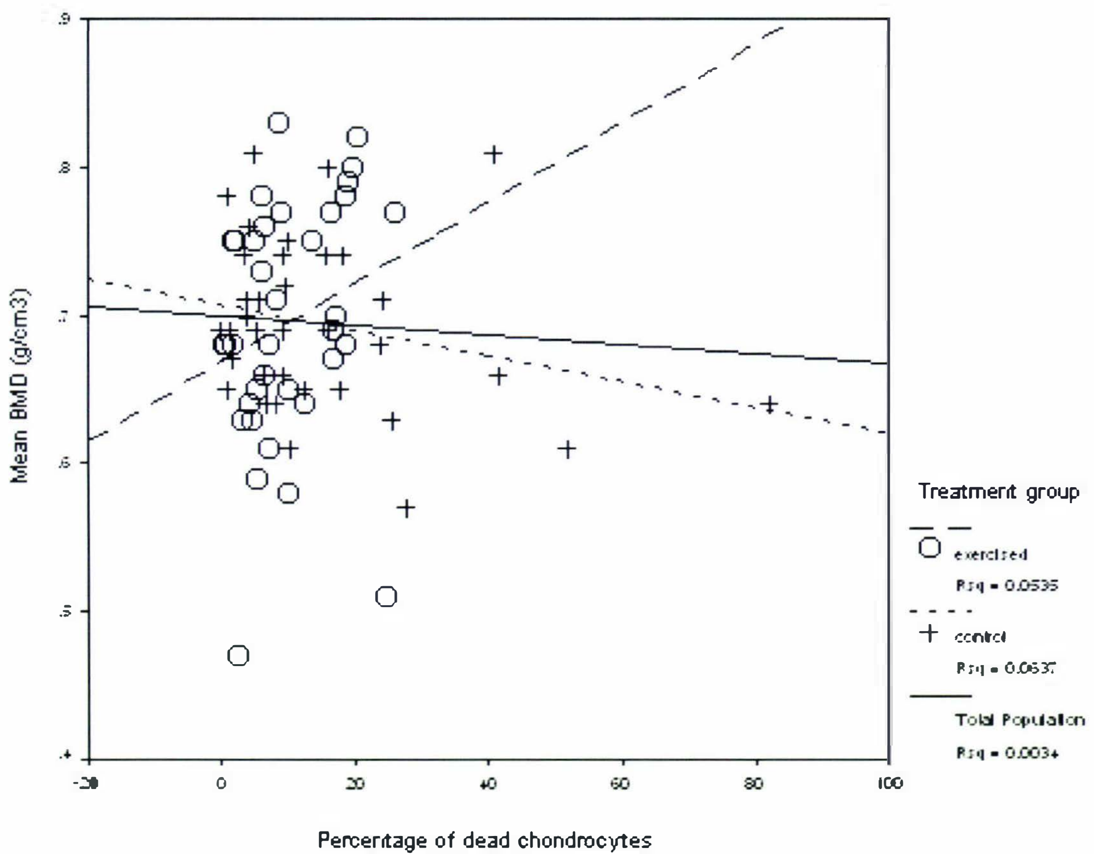


Figure 3.20: Regression of the mean BMD with percentage of dead chondrocytes across pooled exercised and pooled control sites.

There was no significant relationship of % of dead chondrocytes with mean BMD across pooled dorsal and palmar sites $r^2 = 0.0357$ (Figure 3.21).

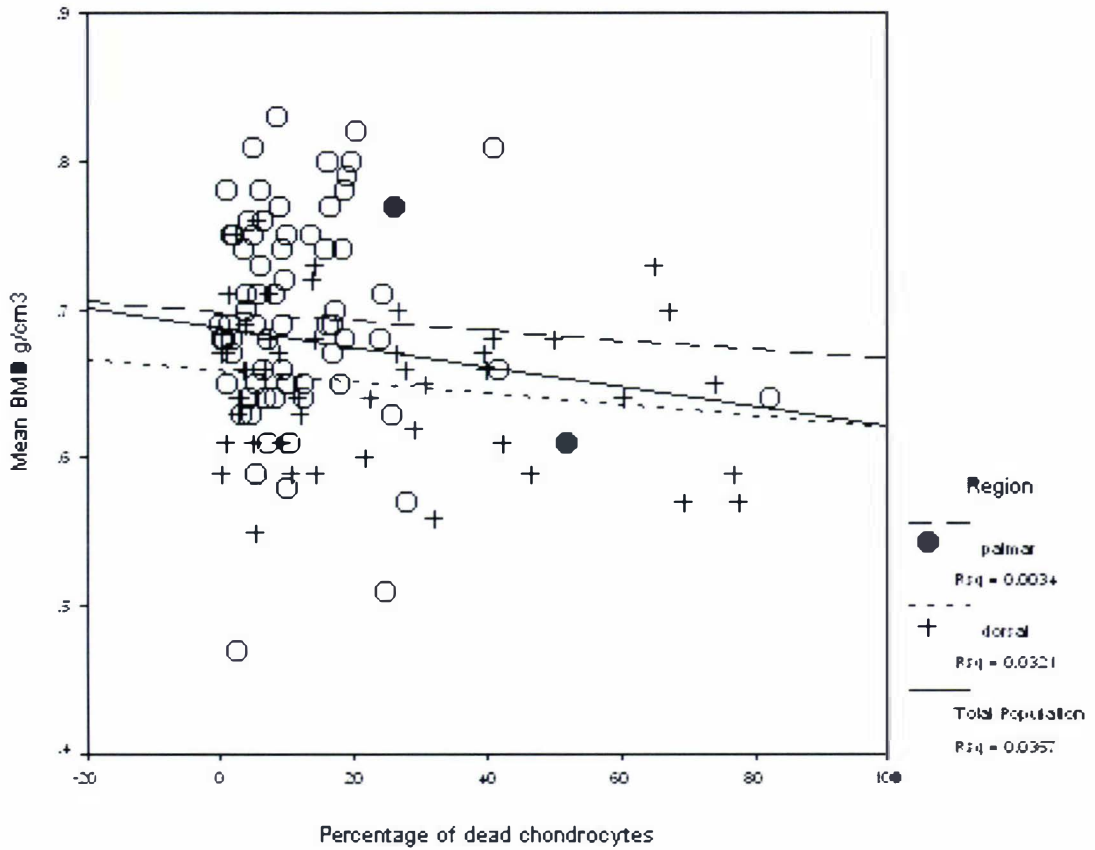


Figure 3.21: Regression of the mean BMD with the percentage of dead chondrocytes across pooled palmar and pooled dorsal sites.

The relationship of % of dead chondrocytes and very high density SCB at ROI

There was no significant relationship of the percentage of dead chondrocytes with the percentage of very high density bone across pooled exercised and control sites, $r^2 = 0$ (Figure 3.22).

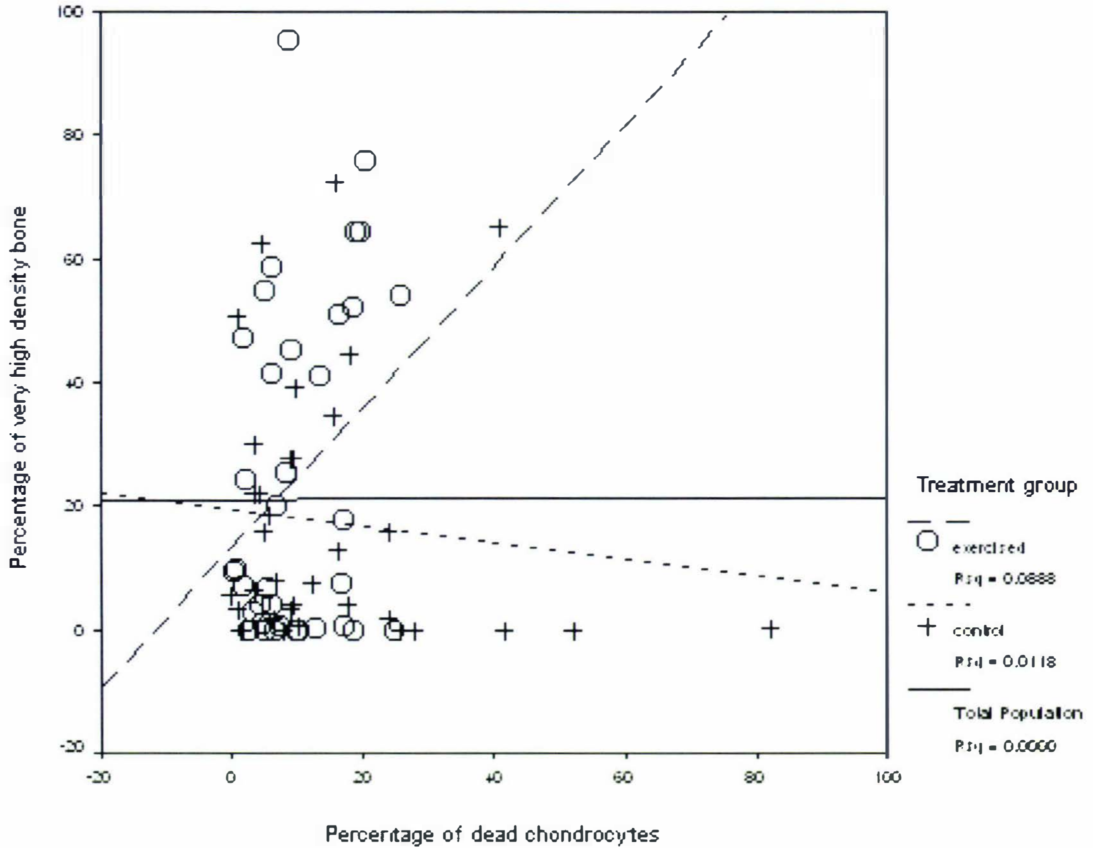


Figure 3.22: Regression of the percentage of very high BMD voxels with the % of dead chondrocytes across pooled exercised and control sites.

There was no significant relationship of the percentage of dead chondrocytes with the percentage of very high density bone across pooled palmar and dorsal sites, $r^2 = 0.0188$ (Figure 3.23).

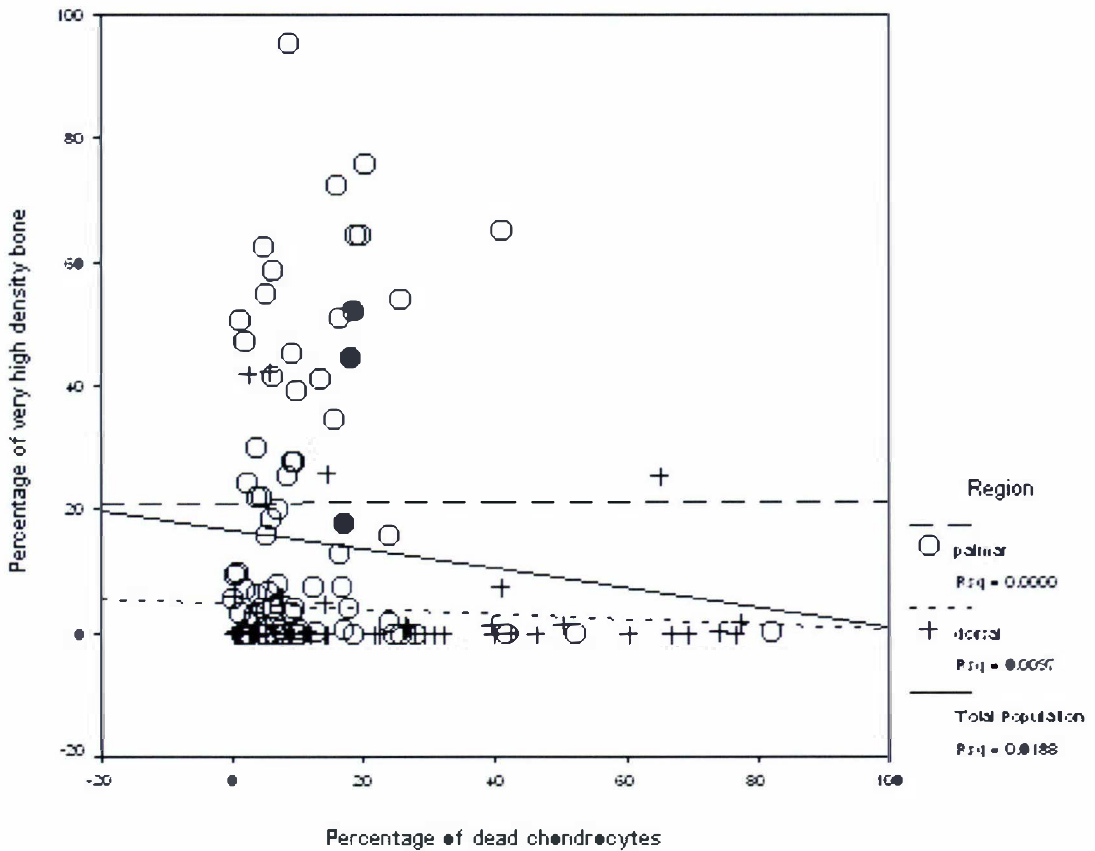


Figure 3.23: Regression of the percentage of very high BMD voxels with the % of dead chondrocytes across pooled palmar and pooled dorsal sites.

There was no significant relationship of the percentage of dead chondrocytes with the percentage of medium –very high density bone for exercised and control groups ($r^2 = 0.0108$) and palmar and dorsal regions ($r^2 = 0.0108$).

3.9 Location of Dead Chondrocytes

Articular cartilage zones A and B

To compare the relative number of dead chondrocytes in the two zones (A – superficial and intermediate and B - deep) the percentage of dead chondrocytes in each zone was expressed as a percentage of the total number of chondrocytes in each zone.

Across pooled sites mean percentage of dead chondrocytes was 133% greater in zone A (25%) than zone B (10.7%) (p= 0.001) (Figure 3.24).

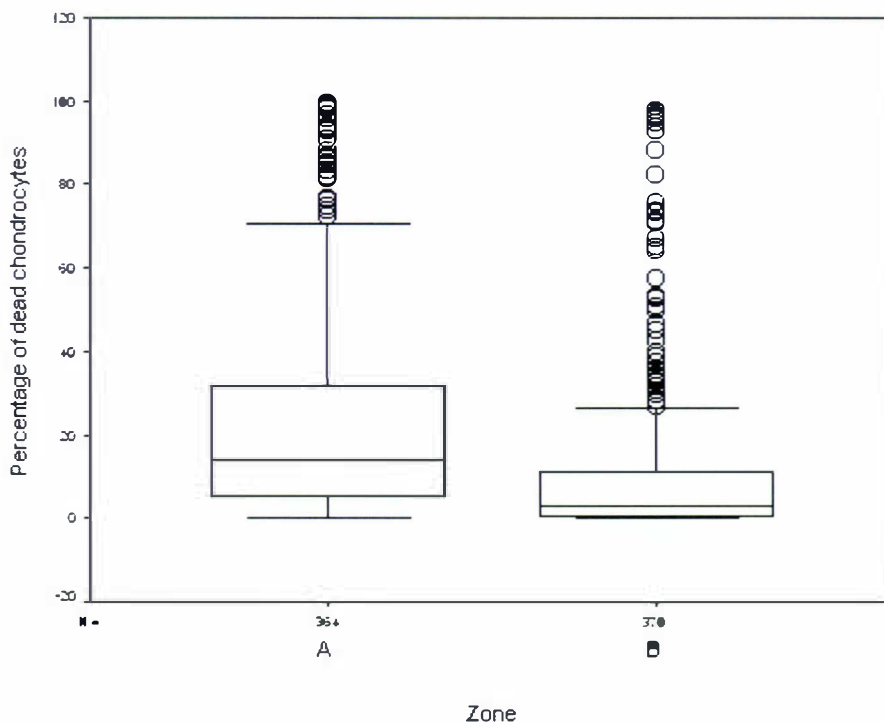


Figure 3.24: Box and whisker plot of the percentage of dead cells in zones A and B of the articular cartilage sections.

A greater percentage of dead chondrocytes was present in Zone A (exercised 16.4%, control 33.5%) than zone B (exercised 7%, control 14.5%) in exercised and control groups (p= 0.003) (Figure 3.25).

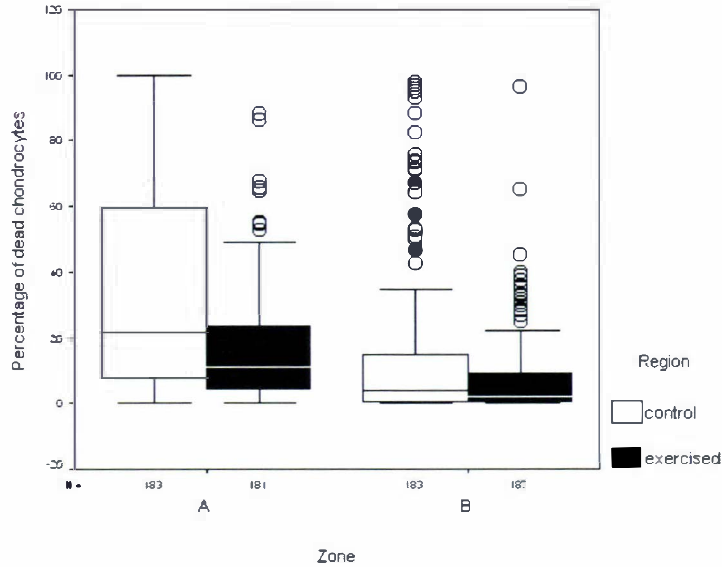


Figure 3.25: Box and whisker plot of the percentage of dead chondrocytes in each zone by treatment group.

A greater percentage of dead chondrocytes was present in zone A (palmar22%, dorsal 30%) than zone B (palmar 6%, dorsal 19%)(p=0.074) (Figure 3.26).

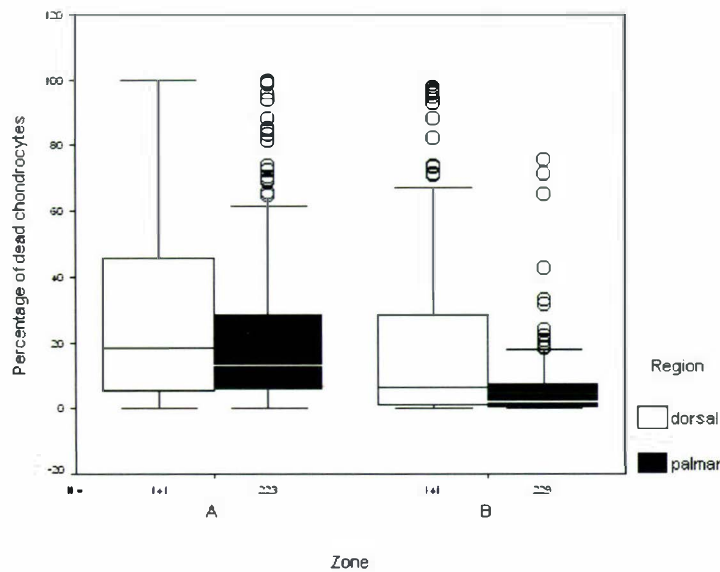
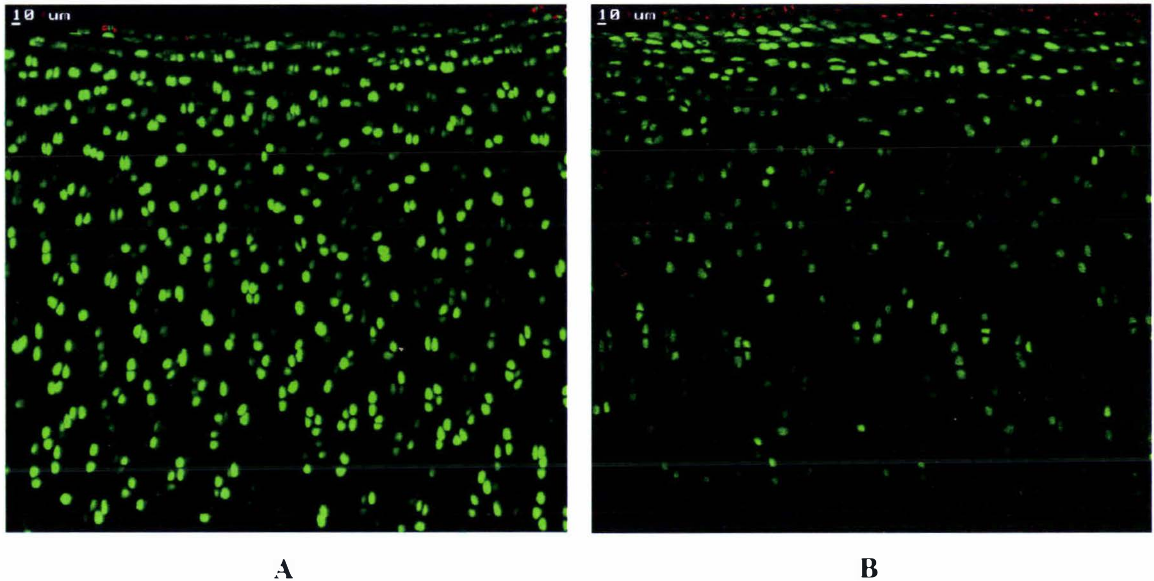


Figure 3.26: Box and whisker plot of the percentage of dead chondrocytes in each zone by region

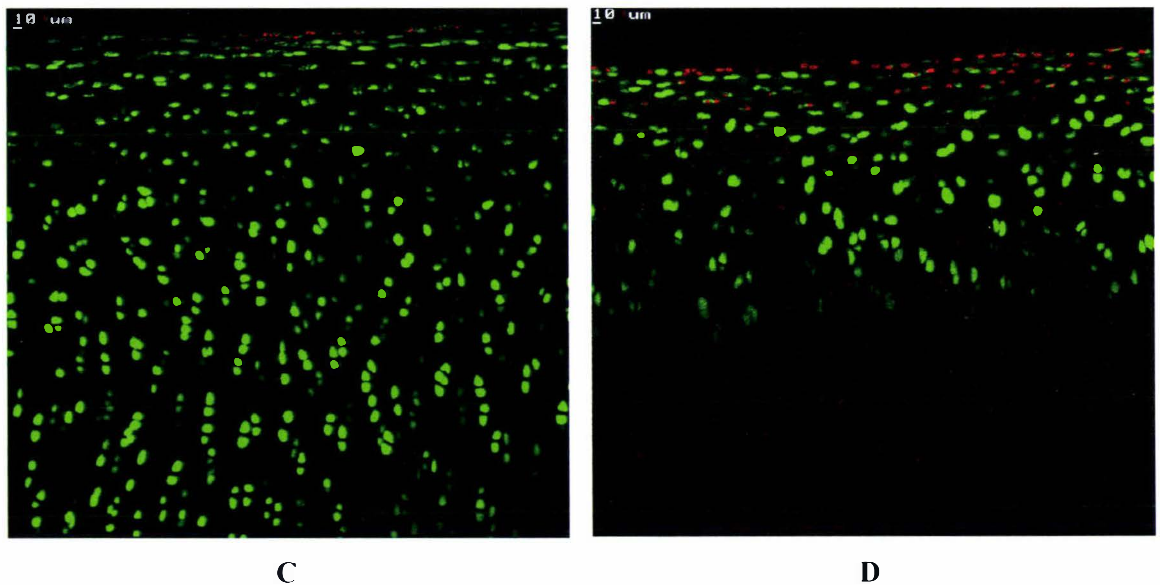
3.10 Examples of Patterns of Chondrocyte Viability Staining

The following are examples of the confocal images of the articular cartilage, from articular surface to tideline. Several repeatable patterns of chondrocyte staining were observed. All images were obtained using the 16x objective and are 625μm x 625μm x 10μm. The articular surface is at the top of the image, the tideline at the bottom. Green staining chondrocytes are classified as viable and red as dead.



A B

Figure 3.27: Confocal images of viable staining chondrocytes.



C D

Figure 3.28: Confocal images of viable staining chondrocytes.

A small number of dead chondrocytes are present at the articular surface. Note the differences in thickness between images (C) and (D). Image (C) is from a dorsal site and image (D) from a palmar site.

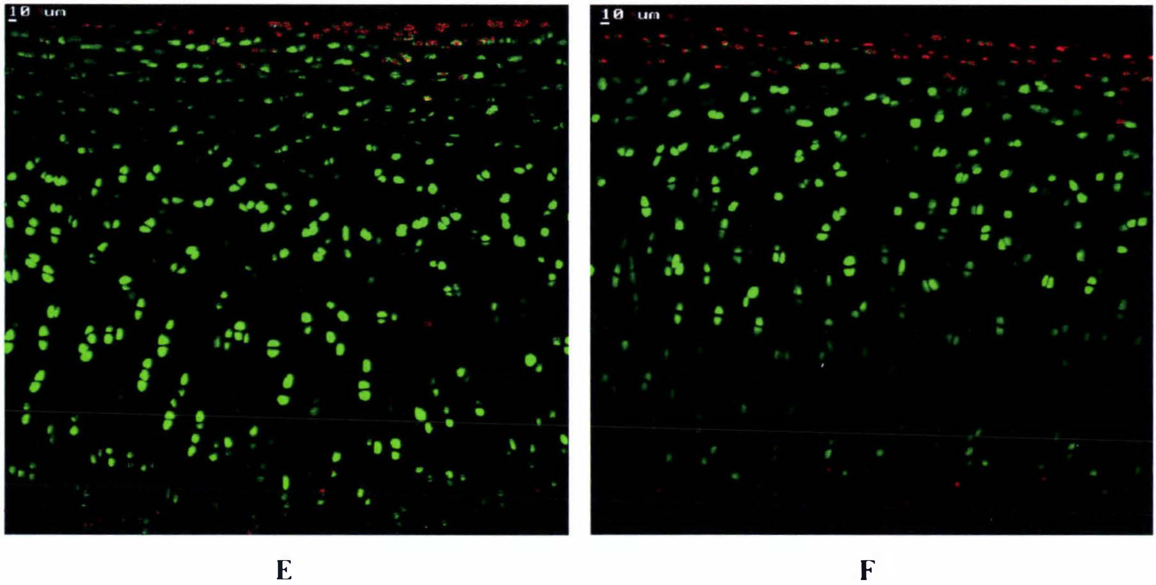


Figure 3.29: Confocal images of predominantly viable staining chondrocytes.

Both images (E) and (F) have an increased number of dead chondrocytes at the articular surface when compared to images (C) and (D). The articular surface is relatively regular.

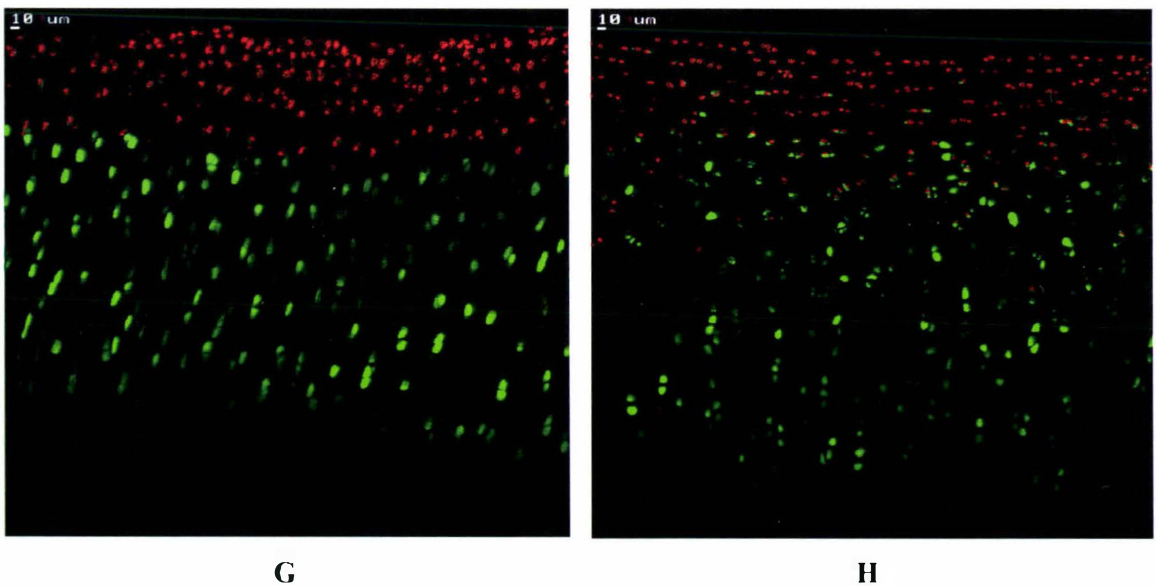


Figure 3.30: Confocal images of viable and dead staining chondrocytes.

An increasing number and thickness of dead chondrocytes is present in images (G) and (H) associated with a more irregular articular surface than images (E) and (F).

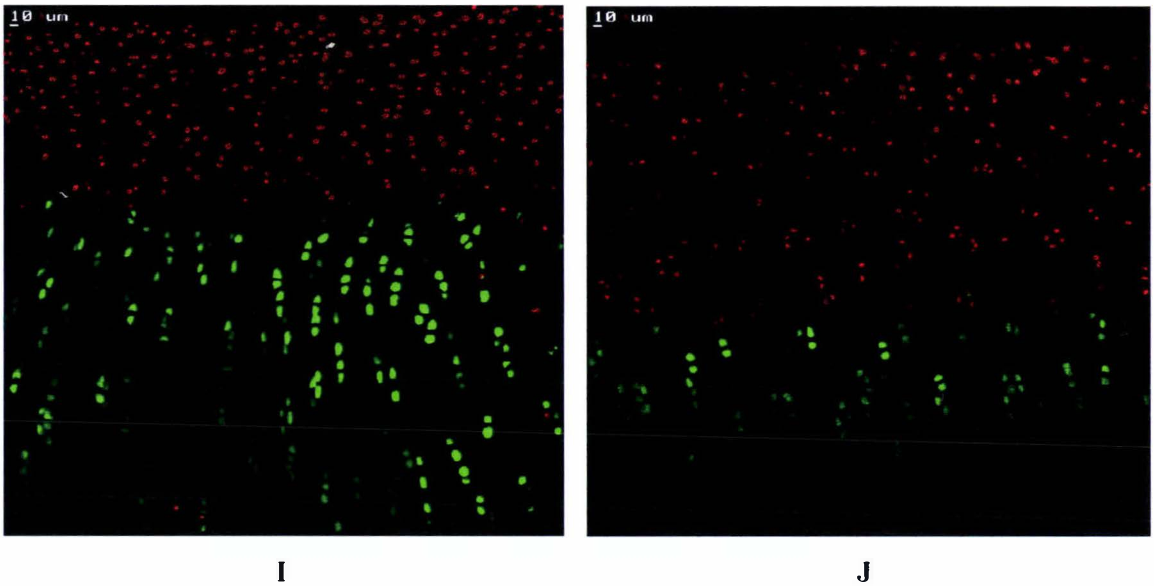


Figure 3.31: Confocal images of viable and dead staining chondrocytes.

An increasingly large number and thickness of dead chondrocytes involving the articular surface and extending towards the tideline are present in images (I) and (J).

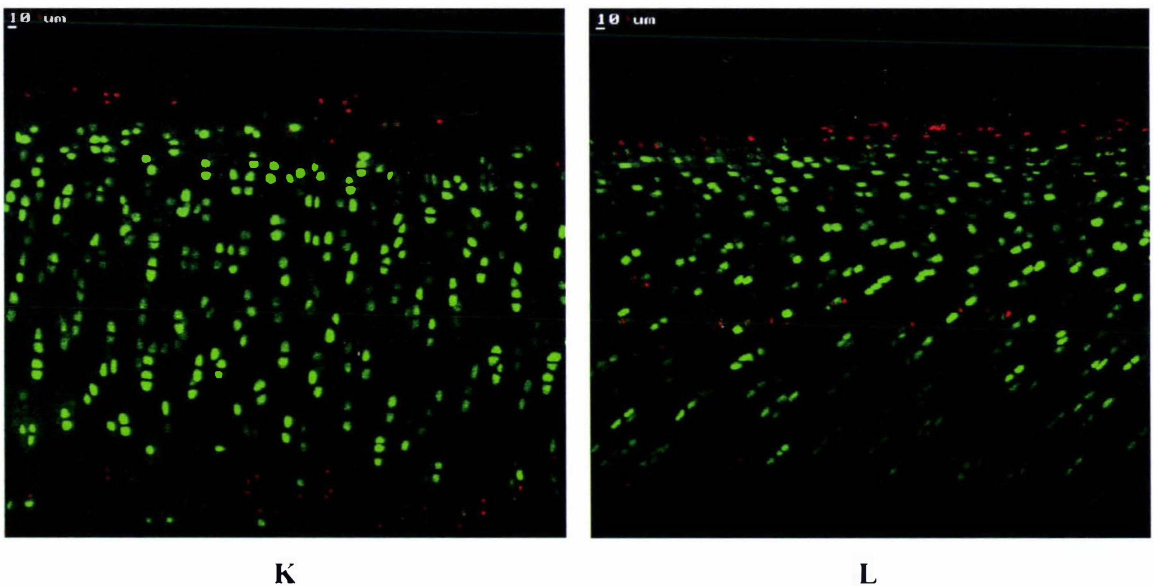


Figure 3.32: Confocal images of viable staining deep zone chondrocytes.

Images (K) and (L) have small numbers of dead chondrocytes at a 'new' articular surface, suspected to be due to superficial zone slough as the chondrocytes are typical of those found in the intermediate and deep zones.

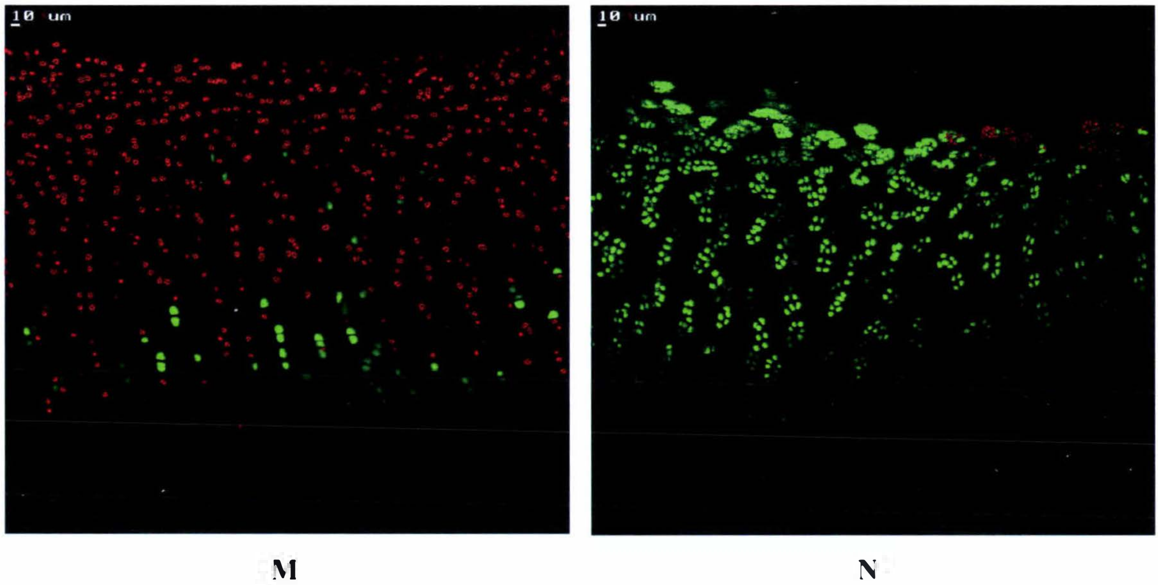


Figure 3.33: Confocal images of abnormal chondrocytes and articular cartilage.

Image (M) has predominantly dead staining chondrocytes indicating severe compromise to cellular function in this location. Images (N) and (O) are examples of osteoarthritic cartilage with an irregular articular surface and marked chondrocyte chondrone formation involving predominantly viable chondrocytes. Image (P) is also from OA cartilage and consists of non-viable cellular debris adjacent to a number of viable, irregularly organised chondrocytes.

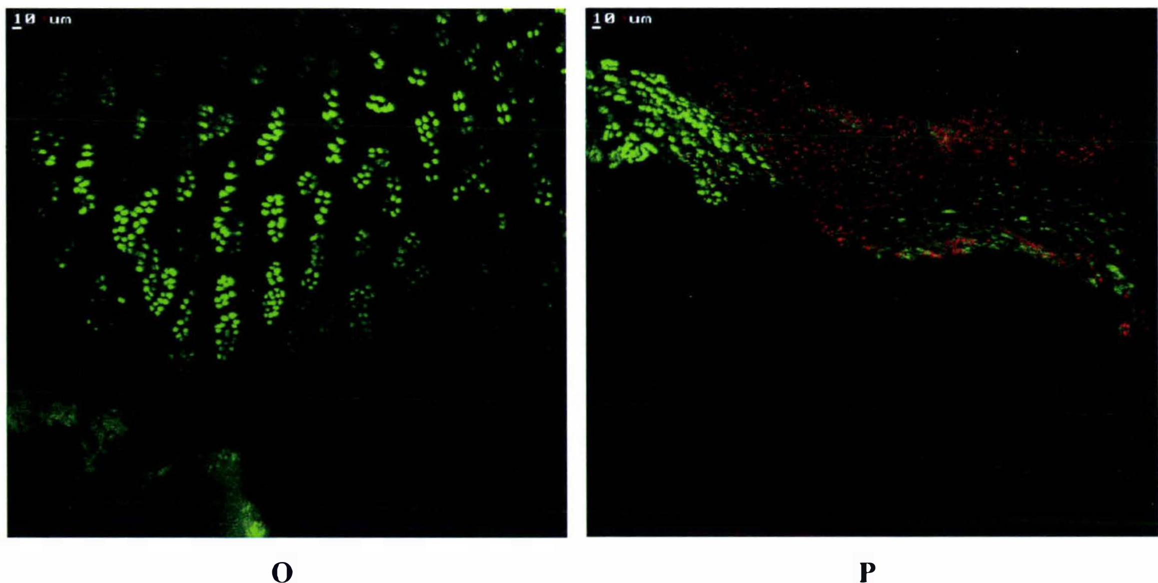
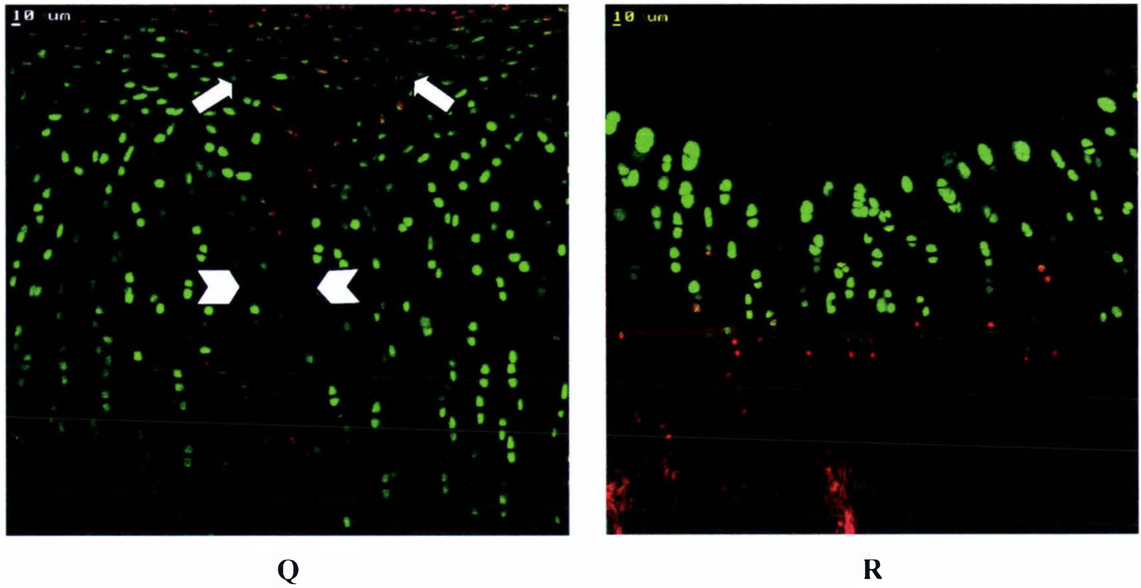


Figure 3.34: Confocal images of abnormal chondrocytes and articular cartilage.



Q R

Figure 3.35: Confocal images of articular cartilage abnormality.

Image (Q) demonstrates a pattern of 'trough' formation of dead chondrocytes in the centre of the articular cartilage (arrow heads) extending from tideline to the articular surface where it widens (solid arrows) and peaks, possible precursor of articular cartilage fissure or wear line. Image (R) was taken perpendicular to a gross 'wear line' in the articular cartilage and demonstrates an irregular articular cartilage surface with large round chondrocytes arranged in chondrones, there are no discernible superficial or intermediate zones present.

3.11 Histological Assessment of Articular Cartilage

Modified Mankin score

The difference between the overall modified Mankin score for control and exercised groups did not reach the significance level (p= 0.069) (Figure 3.36).

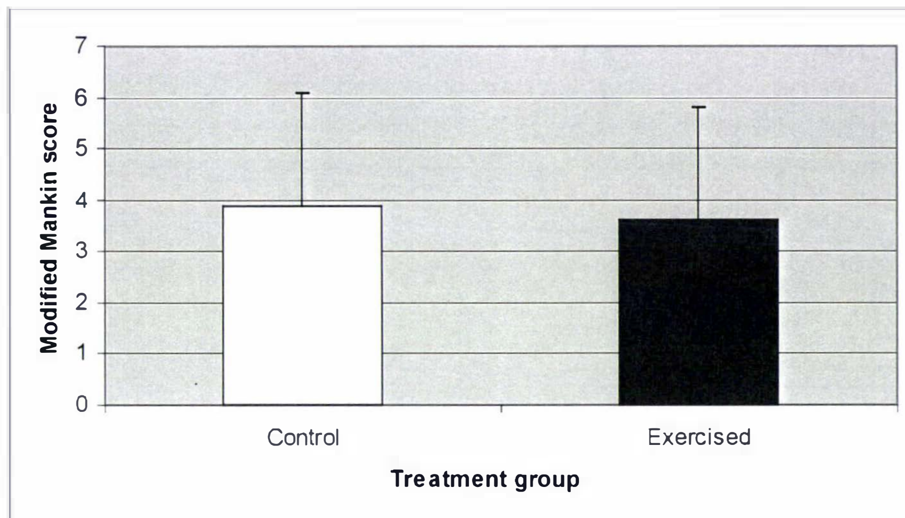


Figure 3.36: Histogram of the modified Mankin scores across pooled control and exercised sites

There was no significant difference in overall modified Mankin score between regions (dorsal vs palmar (Figure 3.37) or lateral vs medial) and no interaction between treatment group and region.

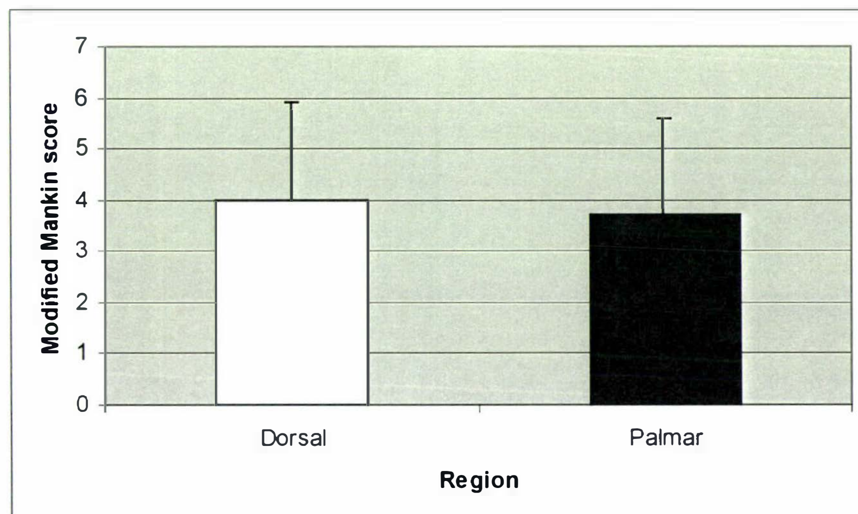


Figure 3.37: Histogram of the overall modified Mankin scores for dorsal and palmar regions.

There was a significant difference in the overall modified Mankin score between colts and fillies (p=0.015). A significant interaction with treatment group was also present (p=0.002) with control fillies having greater modified Mankin scores than exercised fillies, and control and exercised colts (Figure 3.38).

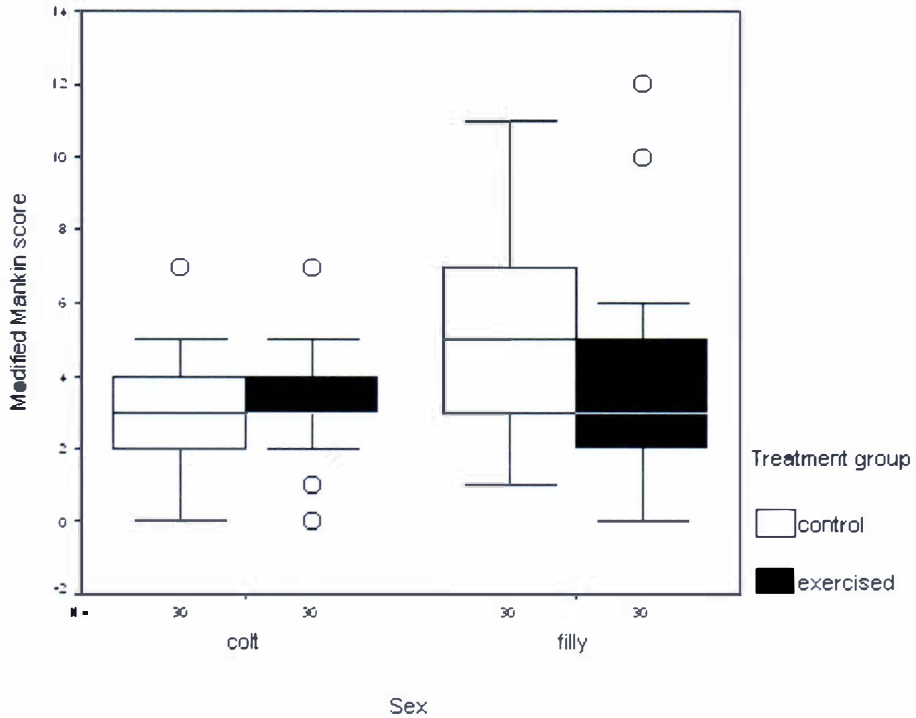


Figure 3.38: Box and whisker plot of pooled colt and filly scores by treatment group

SOFG score

There was a significant difference between SOFG score with the exercised group having a greater overall SOFG score than the control group ($p=0.001$) (Figure 3.39).

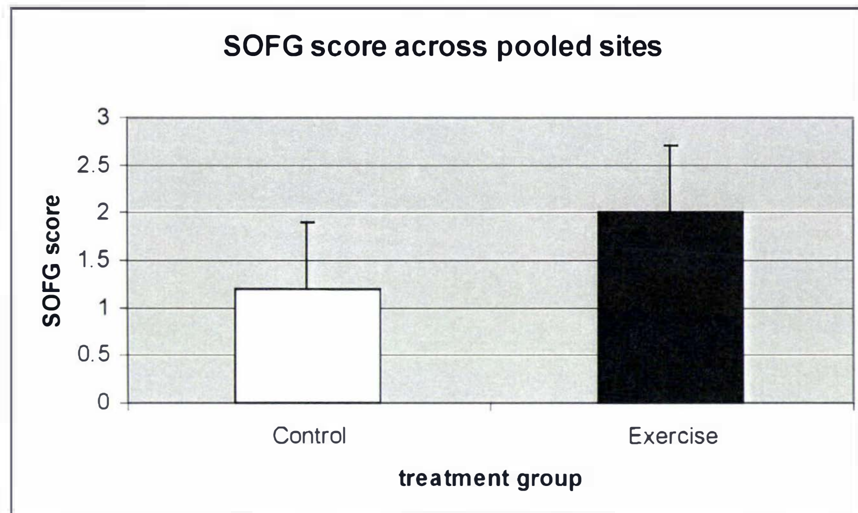


Figure 3.39: Histogram of the overall SOFG score for treatment group

There was no significant difference between dorsal and palmar (Figure 3.40), or lateral and medial regions, nor an interaction with region or sex.

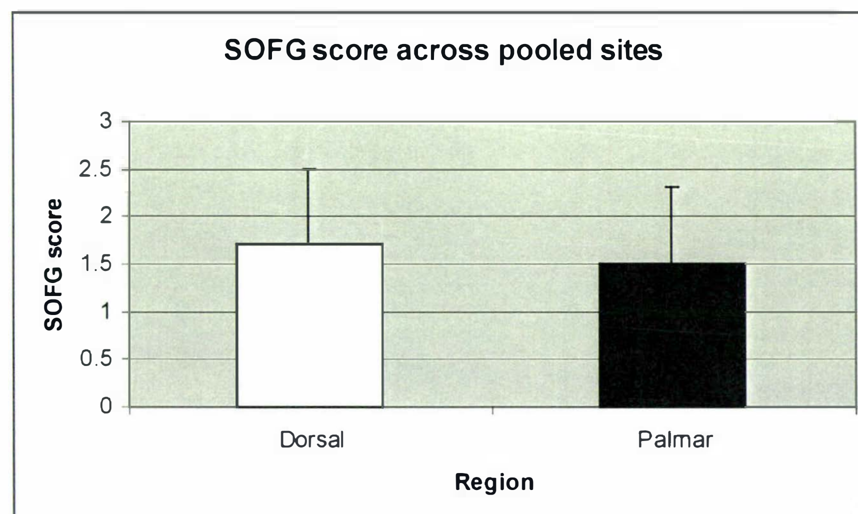


Figure 3.40: Histogram of the overall SOFG score by region.

3.12 Examples of Confocal Images With Corresponding Histological Images

The confocal images (16x objective, left image), and histological images (10x objective, SOFG the middle and H&E the right image), from adjacent cartilage are displayed below.

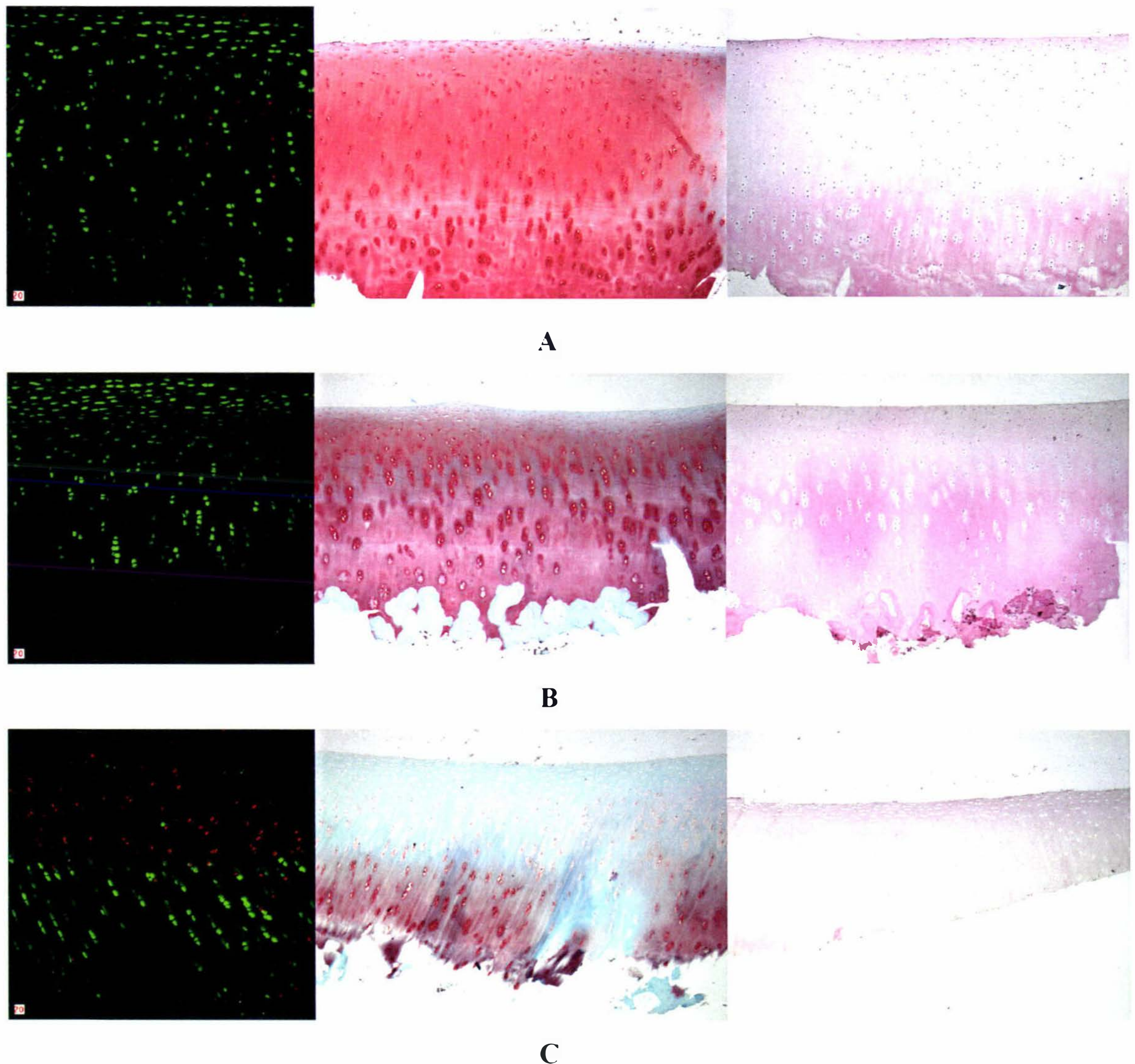


Figure 3.41: Images comparing confocal, SOFG and H&E staining of the articular cartilage.

Images (A) show predominantly viable staining chondrocytes with corresponding SOFG staining of all zones of the cartilage. Images (B) demonstrate predominantly viable chondrocytes with SOFG staining in all but the very superficial zone of the cartilage. Images (C) show a number of dead chondrocytes at the articular surface of the articular cartilage with corresponding reduction of SOFG staining.

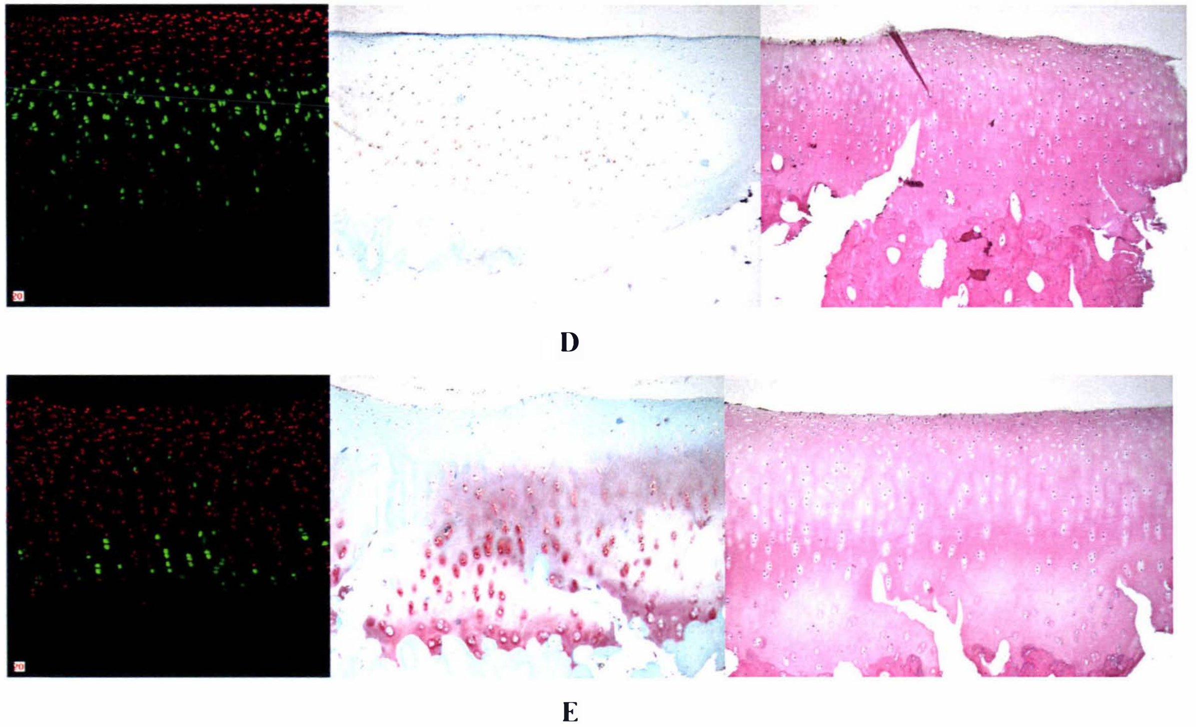


Figure 3.42: Images comparing confocal, SOFG and H&E staining of the articular cartilage.

Images (D) and (E) demonstrate increasing numbers of dead chondrocytes and reduced SOFG staining with surface irregularity present in the histological sections.

CHAPTER 4 DISCUSSION

The objectives of this chapter are to consider the results in the context of the effects of exercise and loading on the articular cartilage and subchondral bone of the distal third metacarpal/metatarsal bones. Articular cartilage viability will be discussed, initially in relationship to the percentage of viable chondrocytes for each treatment group and region, and then in the context of the percentage of dead chondrocytes overlying SCB of known density. The total number of chondrocytes, non-calcified articular cartilage thickness, distribution of dead chondrocytes, and finally, the gross pathologic changes and histological findings will be discussed. The limitations of the findings are presented. In conclusion, the place of this study in existing OA work and possibilities for further research will be discussed. All differences referred to in the discussion, unless otherwise stated, are those shown to be significantly different ($p<0.05$).

4.1 Effect of Exercise and Loading on the Percentage of Viable Chondrocytes

Mean chondrocyte viability (pooled sites) was 14% greater in the exercised compared to the control group, and 16% greater in pooled palmar than dorsal sites.

There were more viable chondrocytes at sites of known or imputed increased articular cartilage stress of distal Mc3, both within joints and horses, and also between treatment groups. The mean percentage of viable chondrocytes at dorsal sites was 34 % greater in the exercised group compared to the control group. Mean chondrocyte viability percentage was 33% greater at the palmar, compared to the dorsal, sites of the control group horses, and may be due to the effect of loading on the cartilage, and of exercise.

It has been previously shown in static loading, biomechanical, biochemical, CT and pathological studies, that palmar sites sustain greater stress than the dorsal aspect of distal Mc3 (Brama et al. 2000). Loading stress within the fetlock joint is possibly greater, and cumulative at (some) higher gait speeds, because of the increased number of gait cycles at the canter or gallop (Nunamaker et al. 1990). The number of higher velocity gait cycles was greater in exercised than pastured horses, although this was not absolutely documented by 24 hour observations over the 18 months of the conditioning study.

Exercise, and site within the joint, significantly affected the variability in the percentage of viable chondrocytes, which was more homogeneous in exercised horses as a group, and in palmar and medial sites, than dorsal and lateral sites, of distal Mc3 of control and exercised horses; variation in chondrocyte viability was greater in the dorsal articular cartilage, particularly if not conditioned by the exercise regimen. Chondrocyte viability was greater and less variable at the medial condylar sites compared to lateral condylar sites. This difference was not as marked as that between treatment groups and between palmar/dorsal regions.

The presence of a regional (palmar/dorsal, medial/lateral) effect supports a loading as well as an exercise effect on the articular cartilage. There was no significant difference between palmar exercised and palmar control sites, indicating that at the palmar sites, loading associated with conditioning, and that associated with pasture exercise, contributed similarly to the homogeneity of the percentage of viable chondrocytes. The effect of exercise and loading on chondrocyte viability and variability is further demonstrated by the significant interaction of palmar forelimb and hindlimb sites with treatment group. The 5% greater mean percentage of viable chondrocytes for forelimb exercised palmar sites compared to hindlimb exercised palmar sites is likely to be due to the greater concussion of the forelimb at hoof impact than the hindlimb (Back 2001).

In the OCD-exercise (EXOC) study (van Weeren 2000), foals up to 5 months of age were confined to stables and received short bouts of vigorous exercise (an increasing number of gallop sprints of 30 seconds to 4 minutes six days per week). Detrimental effects on articular cartilage health were shown compared with box rest or free pasture exercise regimens. Free pasture exercise was later suggested as most suited to articular cartilage development and repair of minor defects (van den Hoogen et al. 1999). In that study the effect of training superimposed on paddock exercise was not addressed. The results of the studies in this thesis show that controlled exercise (daily slow canter of 4.2-6.6 m/s with 250m sprint at peak velocity (12 m/s) after 6 months of age), superimposed on free pasturing had a beneficial effect on the viability of chondrocytes. The presence of a regional effect suggested a loading as well as an exercise effect on chondrocyte viability. Articular cartilage conditioned by moderate exercise from an early age may be better able to withstand the demands placed upon it during pasture exercise.

The difference between the findings after vigorous exercise (EXOC study) and moderate longer duration exercise (the present study), may have been due to one or more of: exercise in horses of less than 5 months of age may have caused cartilage damage that was not apparent at 18 months, the spontaneous pasture exercise may have attenuated the possible damaging effect of the sprinting exercise, differences in exercise working surface, different gait speeds, different

number of cycles or rapidity of the increase in fast workload, or breed difference between young Warmblood and Thoroughbred horses.

4.2 Effect of Exercise and Loading on the Total Number of Chondrocytes

The total number of chondrocytes (live and dead) was corrected to account for the different thickness of the articular cartilage, which was most obvious between dorsal and palmar sites. The total number of chondrocytes was not different between control and exercised horses for pooled sites. There was a 13% difference in mean total chondrocyte number between pooled dorsal (584 chondrocytes/mm) and palmar (661 chondrocytes/mm) sites. There was no difference between medial and lateral sites, nor an interaction of treatment group with region.

The greater mean total number of chondrocytes at pooled palmar sites may indicate a congenital or even peri-natal effect, possibly in response to the higher articular cartilage stress in the palmar region in combination with exercise level, on chondrocyte number. When articular cartilage thickness was not taken into account the mean total number of chondrocytes was 13% greater for control group horses than the exercised group which was found to be due to the regional thickness differences.

There was large inter-horse and intra-horse variation in the total number of chondrocytes (Figure 3.4). As each individual has its own articular cartilage genotype, variation in the total number of chondrocytes within, and between, individual horses is perhaps not surprising. Historically there is thought to be minimal change in the absolute number of chondrocytes during maturation (Stockwell and Meachim 1979). An apparent partial reversion to embryonic phenotype occurs in mature human articular cartilage exhibiting signs of OA but even in diseased articular cartilage the change in total number of chondrocytes is not dramatic (Sandell and Aigner 2001).

Due to the large variation, difference in chondrocyte number is difficult to interpret in relation to articular cartilage "quality". A difference in the total number of chondrocytes between control and exercised horses would not be expected in post-natal articular cartilage. Differences in total chondrocyte number between palmar and dorsal pooled sites may be due to one or more of: intra-uterine or post-natal factors determining chondrocyte number, or to the ability, although limited, of chondrocytes in skeletally immature and mature individuals to differentiate (Matyas et al. 1997; Pullig et al. 2000) and divide (Sandell and Aigner 2001).

Any difference, or increase, in chondrocyte number would be expected to be small with the mitotic response reported to be limited in mature animals. However, in animals of this age group (<18 months) there may be a greater capacity for chondrocyte division in response to stimuli. Based on the total number of chondrocytes alone it is not possible to determine if the observed differences in total chondrocyte number are pathological or physiological, however the differences observed in palmar and dorsal sites are likely to be physiological.

The total number of chondrocytes and the DNA content of articular cartilage were assessed in middle carpal joints of intensely exercised and less intensely exercised horses (Murray et al. 2001). The highly loaded dorsal carpal sites had significantly more chondrocytes than did less loaded sites, overall and within each exercise group; chondrocyte number was not significantly different between the two exercise intensities (Murray et al. 2001). The higher articular compressive stress in dorsal regions of the third carpal bone (Firth and Hartman 1983; Bramlage et al. 1988; Firth et al. 1999) may influence the greater chondrocyte density in the dorsal sites of the middle carpal joint, and might be extrapolated to the palmar MCP/MTP sites in this study, which are also imputed to sustain higher articular stress. However, comparing the results of the two studies requires caution as there were differences in the age of the horses, joint assessment method and exercise intensity and type.

4.3 Relationship of Chondrocyte Viability and SCB BMD

The SCB (consisting of the subchondral cortical bone-plate and subchondral epiphyseal bone) was measured at ROI of 6mm x 6mm x 2mm, placed 2mm proximal to the junction of articular cartilage and subchondral bone, directly beneath the articular cartilage sample sites. The ROI was so positioned that the normal high density of the calcified cartilage, and so-called subchondral bone-plate, would not conceal possibly subtle changes in the bone just deep to these tissues. Initially a square of 3mm x 3mm in a 2mm thick CT slice was used to assess SCB BMD. No difference between exercised and control group BMD was detected. It was thought that the ROI may have been mostly contained within the subchondral bone plate, incorporating minimal subchondral epiphyseal bone and not reflecting the response of the SCB to exercise or loading. Using a larger ROI of 6mm x 6mm x 2mm at the same location as the smaller ROI as described above, differences between palmar and dorsal region SCB were demonstrated, but treatment group differences were still not significant.

The density of the SCB differed between sites. The percentage of very high density bone within the ROI was 5.5 times greater in the palmar than dorsal sites of both the exercised and control

horses. However mean BMD, and percentage of very high density bone of the ROI, were not significantly different between exercised and control groups for pooled forelimb sites, which might indicate that exercise intensity was not vigorous enough to induce the significant bone response hypothesised, at least in the site of the ROI. Pasture activity and play alone may have been sufficient to induce SCB (re)modelling similar to that in the exercised group. The number of strain cycles to induce bone formation is not high (Cornelissen et al. 1999), and foal play activities at pasture may produce enough high strain, high strain rate cycles; the superimposition of exercise at submaximal velocities may not have produced "novel" strain (Lanyon and Skerry 2001) sufficient to induce further SCB (re)modelling.

There was no relationship between the mean BMD or percentage of very high density bone and the percentage of dead chondrocytes. Because sprint training has a larger effect on SCB BMD than pasture exercise (van Weeren 2000), speed, impact and strain rate may be more important than duration of exercise for BMD changes (Young et al. 1991b; Firth et al. 1999).

The amount of very high density bone in the exercised horses indicates that the stimulus provided in the exercise programme resulted in some (re)modeling, at least in terms of the definitions used to expose sclerosis, and the methods available to us to quantify it. The inability to demonstrate a relationship between the percentage of dead chondrocytes and increasing sclerosis in the SCB sites examined may be due to one or more of the following reasons: the degree of SCB sclerosis may not have been close enough to the density required to be detrimental to the overlying articular cartilage, or the load dissipated through the joint not of sufficiently high stress to damage the articular cartilage, even if SCB was of sufficient density to cause cartilage compromise. It appears that in this study articular cartilage compromise, as assessed by chondrocyte viability, was not related to SCB sclerosis.

4.4 Location of Dead Chondrocytes Within the Articular Cartilage

The articular cartilage closest to the articular surface contained the greatest percentage of dead chondrocytes. This pattern of increased percentages of dead chondrocytes in the superficial and intermediate zones, for example in Figure 3.30 and Figure 3.31, relates to the observed superficial zone loss in a number of confocal images (Figure 3.32). Perhaps the dead chondrocytes are an early sign of idiopathic OA, since in human articular cartilage affected by OA there is a strong positive staining for IL-1 in the upper half of the articular cartilage, in both chondrocytes and matrix (Blanco Garcia 1999).

If SCB sclerosis was linked to the initiation of cartilage damage or chondrocyte apoptosis one might expect the chondrocyte death to occur at the tideline or cartilage-SCB interface. However, the results of this study showed chondrocyte death was predominantly associated with the superficial and intermediate zones of the articular cartilage (Figure 3.30 and Figure 3.31). It appears that the distribution of dead chondrocytes is not SCB related, supported by the lack of association between percentage of dead chondrocytes and SCB sclerosis.

Studies using human osteoarthritic cartilage (Heraud et al. 2000; D'Lima et al. 2001b) and bovine cartilage explants (wounded centrally with a trephine and maintained for up to 10 days) (Tew et al. 2000), support the finding that chondrocyte death begins at the superficial zones of the articular cartilage. It has been suggested that absence of superficial articular cartilage may be early change in OA (Mitchell et al. 1992). The confocal images of articular cartilage obtained in this study showed patterns suggesting a continuum of matrix degeneration secondary to superficial chondrocyte death and sloughing. Viable staining chondrocytes typical of those from the deep zone were found exposed at the "new" articular surface (Figure 3.32), further supporting, and consistent with, the dead superficial chondrocytes at an earlier stage of a progressive process.

4.5 Effect of Exercise on Non-calcified Articular Cartilage Thickness

A difference in non-calcified articular cartilage thickness between control and exercise groups was not apparent until examined by region. The exercised palmar sites were thinner when compared to dorsal sites (Figure 3.28), either exercised or control. This thickness difference may be due to regional differences in loading within the joint, the thinner articular cartilage in the palmar aspect of the condyles being related to higher stress (Vilar 1995). In severe OA non-calcified articular cartilage thickness may increase as a result of up-regulation of the anabolic processes of chondrocytes and water imbibition, which may explain the thickened cartilage in palmar sites of the left hindlimb of G20, up to 1.2mm, in which there were gross, histological and confocal changes consistent with OA.

4.6 Patterns of Cell Staining

Several repeatable patterns of chondrocyte staining were observed. Apparent 'dual' staining with both calcein and PI was observed. Reported in rabbit physes, dual staining is possible in viable chondrocytes with a defective cell membrane, and likely indicates very early cell

compromise (Roach and Clarke 2000). In studies on sperm viability, dual staining cells have been described as a transitional population of cells that have lost some functional capacity as they are not able to resist uptake of PI (Collins and Donoghue 1999). Another possibility is artefact due to the close proximity of a viable and dead chondrocyte in different planes, which appear as one cell. This is unlikely, as the average size of the stained chondrocyte was similar to the 10 μm thickness of the image examined, making the chances of superimposition low.

The size of calcein-stained viable chondrocytes varied in some sites. While most deep zone chondrocytes were round and plump, occasional samples had smaller chondrocytes, which may indicate a reduction in chondrocyte volume, with membrane compromise but maintenance of functional organelles. This is consistent with a theory of a continuum of chondrocyte viability with varying effectiveness of metabolic processes. For the purposes of this study classifying chondrocytes as either viable or dead was sufficient. Dual staining chondrocytes were counted as both viable and dead. The number of dual staining chondrocytes was low and would not have affected the results. To further investigate and validate chondrocyte viability as indicated by calcein, quantitative assessment of chondrocyte metabolism would be necessary.

In a number of fields, the superficial and/or intermediate zones were absent, with viable round chondrocytes at the "new" articular surface organised in a random to perpendicular fashion (Figure 3.32). The absence of the superficial zones may be related to early superficial surface erosion, fibrillation or wear lines. Even in severe OA (Mankin scores greater than 8) chondrocytes of the middle and deep layers are reported to keep their anabolic capacity (Sandell and Aigner 2001). Dead chondrocytes are likely to remain trapped in ECM initially but as the matrix becomes further compromised it may be removed with the apoptotic or necrotic chondrocytes which once maintained it. As cells die and slough, the deeper chondrocytes are exposed and left to attempt to repair the matrix in a limited way, by one or more of up-regulation, changing the type of GAG produced or dividing to form chondrones (Sandell and Aigner 2001).

Light microscopy and ultra-structural observations of wear lines indicate that the chondrocytes of the superficial layer of the affected articular cartilage are necrotic and that there is loss of PG as assessed by safranin O/fast green staining depletion (Brama et al. 2000a). Findings from the present study support that wear lines occur due to apoptosis or necrosis of the superficial chondrocytes and when this layer is sloughed then viable deep zone chondrocytes forming chondrocyte clusters are present in a trough like formation (Figure 3.35).

4.7 Medical History and Gross Pathology

The medical history of the animals revealed only one horse (G20) to have had possible MCP/MTP joint related injury or lameness in the six months prior to euthanasia. Of the six horses examined in the six months prior to sampling, four were exercised and two were controls. Of the six horses with gross evidence of MCP/MTP joint pathology at euthanasia, five (83%) were control horses.

Our findings are in contrast to a study of seven two year old Thoroughbred horses participating in a 13 week exercise programme typical of training of young New Zealand Thoroughbreds (Brama et al. 2000a), in which wear lines and articular cartilage changes in the MCP joints and alterations in articular cartilage collagen were significantly more severe in the exercised than in the 7 controls raised at pasture and kept in pasture yards during the study. The difference in the findings of the effects of exercise on fetlock joint cartilage may be due to differences in type, intensity and duration of the exercise, the composition of the articular cartilage and age of the horses prior to commencement of the imposed exercise regimen, or perhaps a combination of the imposition of race training after conventional pasture management during development, when pasture management alone was shown in the present study to be associated with lower chondrocyte viability than when combined with an exercise programme.

With the exception of horse No. 20, the horses examined by a veterinarian for lameness in the 6 months prior to euthanasia were not the horses found to have macroscopic and microscopic articular cartilage damage in the fetlock joints. The majority of the evidence of articular cartilage damage, detected in this study by confocal microscopy, was characterised by chondrocyte death or compromise and was likely to have been involved in the very early stages of articular cartilage injury, degeneration, or OA. As early articular cartilage compromise is likely to be clinically silent until gross osteochondral damage results in detectable clinical signs of pain and effusion, chondrocyte death must temporally precede clinically evident chondro-osseous damage and synovial inflammation leading to clinically evident joint disease.

4.8 Histomorphometry, Histochemistry and Confocal Microscopy

The confocal microscopy technique described has been shown to work with equine articular cartilage. Confocal microscopic assessment of chondrocyte viability demonstrates chondrocyte live/dead status, but does not assess the quality, quantity, or type of matrix macromolecules

produced. Histomorphometry and histochemistry are commonly used to assess articular cartilage ECM, and were used in this study (Figure 3.41 and Figure 3.42).

There was no difference in the modified Mankin scores (pooled) of control and exercised group. No interaction with region was present. Histochemical scores in exercised horses were better than control horses for pooled sites. This finding is supported by work showing GAG content is greater both in equine articular cartilage in response to exercise (Brama et al. 1999b; Murray et al. 2001), and in the intermediate and deep zones of beagle articular cartilage after moderate running exercise (Kiviranta et al. 1988). There was no difference in the SOFG score between regions or sex.

The reliability of the Mankin score for OA has been assessed for intra-observer and inter-observer agreement and was found to be an adequate histopathological tool (van der Sluijs et al. 1992). Improved assessment of chondrocyte abnormalities, in particular apoptosis or necrosis may be achieved with use of vital staining techniques. Studies have shown the extent of thermal damage observed on confocal microscopy to be much greater than was evident on histological analysis which underestimated the damage to chondrocytes in laser-induced damage to bovine articular cartilage (Mainil-Varlet et al. 2001). All this suggests that confocal microscopy viability staining is a more sensitive indicator of chondrocyte viability when compared to conventional histological methods, particularly histomorphometry, as chondrocyte viability is not reliably appreciable on histochemical sections due to most tissue preparation processes.

4.9 Workload, Sex and Limb Effects

Fillies had a higher average modified Mankin score than colts, irrespective of treatment group. The higher modified Mankin score of fillies is consistent with the lower percentage of viable chondrocytes found in fillies compared to colts. Cartilage is a sex-hormone-sensitive tissue with human epidemiological studies demonstrating a higher prevalence of OA in post-menopausal women (Cooper et al. 1996; Wluka et al. 2001). The role of oestrogen in the pathogenesis of OA remains controversial (Turner et al. 1997) with both increased (Sahyoun et al. 1999) and decreased (Dennison et al. 1998) prevalence of OA in women receiving hormone replacement therapy. It may be possible that fillies are more susceptible to exercise-induced articular cartilage damage, which may be oestrogen related. Further research may help determine if fillies are more prone to developing OA during training than colts and if so the time that they may be more susceptible ie: pre or post onset of puberty.

The mean total number of chondrocytes was greater in the left forelimb than the right forelimb. Laterality or 'leggedness' has been previously described in horses (Clayton 1994). The dominant limb may be subjected to altered forces, even with frequent changes in direction during exercise. However, as the percentage of viable chondrocytes was not significantly different between left and right forelimbs, and for the reasons discussed in section 4.2, the difference in total chondrocyte number may be an anomaly of the data, and is of questionable biological significance.

4.10 Initiating Event in Osteoarthritis: Articular Cartilage Matrix Degradation or Subchondral Bone Sclerosis?

The results of this study suggest that damage to the superficial zone (represented by Zone A), preceded SCB sclerosis, since there was no relationship of SCB BMD with a range of indices of viability of chondrocytes in any zone. This is supported by other studies implicating primary articular cartilage damage at the articular surface as the initiating event in OA (Howell et al. 1976; Stephens et al. 1979b; Dedrick et al. 1993; Yamada et al. 2002). Articular cartilage changes have been shown to progress in an animal menisectomy model in the absence of a simultaneous increase of the BMD at corresponding sites (Messner et al. 2000). In a canine cruciate transection model of OA, articular cartilage changes were noted 3 months after ligament transaction, but increased thickness of the subchondral plate was not noted until 54 months (Brandt et al. 1991; Dedrick et al. 1993).

In the present study articular cartilage changes were observed in the absence of SCB sclerosis, leading to the conclusion that increased SCB sclerosis may not be necessary for development of changes which may proceed to OA. Although not shown to be OA (since neither gross appearance or ultimate clinical/pathologic change outcomes were examined in this study) the changes seen in some sites certainly appeared consistent with what might be expected in the very earliest stages of OA as previously described. Ideally the relationship between the progressive changes in articular cartilage and underlying tissues should be carried out using each animal as its own control in serial assessments of relevant parameters, but this was not possible in the present study. However the way is open to serial assessments, as sophisticated imaging technologies and innocuous microsampling techniques become available.

4.11 Apoptosis or Necrosis?

Although this study did not determine the nature of the chondrocyte death observed, whether apoptosis or necrosis is involved in chondrocyte compromise is important. Necrosis is mostly irreversible, and as apoptosis can be regulated, its existence would open possibilities for therapeutic intervention in cartilage repair and post-traumatic cartilage lesions (Colwell et al. 2001). Finding that soluble intercellular signalling is involved in the spreading of cell death through the articular cartilage matrix may represent a potential intervention in the pathway (Levin et al. 2001). For treatment purposes, if attempting to influence the death (necrosis or apoptosis) of chondrocytes, disease modifying agents are more easily administered to contact the articular surface. Therefore the possibilities are greater for therapeutic intervention if chondrocyte death occurs at the articular surface as apposed to the interface with the SCB.

4.12 Sources of Error and Limitations of the Study

The main limitation of this study was that the chondrocytes were assessed at one point in time, so that it is difficult to conclusively determine the significance of the findings in terms of physiological and/or pathological progressive changes. Although the sampling sites were based on known and imputed loading of the MCP/MTP joints, the size of the samples was small relative to the size of the joint. Although sample size of treatment groups was small, the power of the study was large with at least 30 confocal images counted for each animal.

In assessing viability of the chondrocytes there was the possibility of artefactual cell death. It has been shown in previous studies that chondrocytes close to a cut surface retained their integrity (Bush and Hall 2001b). We observed cell death at a constant distance (20μm) from the cut surfaces, and used criteria to avoid such artefact.

The time of exposure of the articular cartilage to the viability stains was limited to the 30 minutes required to achieve adequate fluorescence, so as to limit the time the chondrocytes were exposed to potentially cytotoxic DMSO that was used in the calcein stain. Short term intra-articular DMSO does not produce adverse effects on equine articular cartilage or synovium as detected by histological assessment (Welch et al. 1989). If contact with DMSO did increase artefactual cell death, all samples would be expected to be equally affected.

As the technique has been used successfully on articular cartilage from other species, the staining technique was assumed to stain all of the chondrocytes present in the sections. However

this was not validated due to the time constraints of the study, and is a future area for development of the technique. 'Ghost' chondrocytes with partial or incomplete stain uptake were not seen.
The assessment of gross joint damage was by subjective visual examination, as quantitative whole joint OA scoring by Indian ink staining was not carried out due to the necessity to preserve the state of the joint surface for other studies.

4.13 Further Research

Loading of the equine fetlock joint has been investigated in vitro, however in vivo knowledge of joint loading encompassing environmental conditions would be of use. Further research, using differing exercise regimens and management combinations, would be of benefit to study the long term effects of early exercise. To further investigate the effect of exercise and loading on articular cartilage and bone, other sites such as the proximal P_p, PSB or other joints such as the antebrachiocarpal or middle carpal joint could be assessed using chondrocyte viability staining techniques.

Further investigation comparing viability staining with traditional histological methods would be advantageous. Validation of the viability staining technique with histological techniques may further improve the assessment of articular cartilage ex-vivo. Dual staining chondrocytes could be further assessed using high power confocal depth scans to show the location of the stains within the cells. The effect of changes in chondrocyte morphology on the loss of mechanical resilience of articular cartilage in OA was beyond the scope of the project but is an avenue for further research in equine osteoarthritis.

In this study a small ROI of subchondral epiphyseal bone was used and further research could look at the relationship of ROI of different sizes and locations on the relationship with the overlying articular cartilage viability. Larger ROI may have detected a relationship between chondrocyte viability and SCB sclerosis. Although no relationship between chondrocyte death and SCB sclerosis was found in this study, it is possible that within each treatment group there may be individuals in which there was an association between chondrocyte viability and SCB BMD, this is an area for further investigation. It is likely that a combination of events occurs during the initiation of cartilage injury. Further research is necessary to determine the relative contributions of each tissue to the progression of OA. If the primary initiating events leading to OA are fully understood, therapy could be targeted at the initiator, whether that be the chondrocyte or SCB.

4.14 Conclusion

As mechanical loading and exercise have been shown to be detrimental to articular cartilage in equine studies (Murray et al. 2000; Brama et al. 2000a), it had been hypothesised in this study that early exercise would compromise articular cartilage quality, and that due to SCB sclerosis chondrocyte viability would be affected at the junction of articular cartilage and SCB. However the hypotheses were not upheld. An increase in the percentage of viable chondrocytes was found at distal Mc3/Mt3 sites in exercised horses, and the majority of the dead chondrocytes were found to be in the superficial and intermediate zones of the articular cartilage with no relationship to the subchondral BMD.

Early exercise of the level used in this study, superimposed on pasture management, is beneficial to chondrocyte viability and articular cartilage quality. Although it is not possible to draw conclusions from this study as to the eventual consequences for later athletic performance, articular cartilage conditioned by early exercise may enjoy longer term benefits, with an ability to better withstand the demands placed upon it.

The exercise regime employed in this study was not sufficiently different from the loads encountered during free pasture exercise to stimulate a significant difference in the SCB mineral density. Because no relationship was found between chondrocyte death and SCB sclerosis, it is suggested that articular cartilage compromise occurs in the absence of SCB sclerosis.

The knowledge base of the relationship between degeneration of articular cartilage, subchondral bone sclerosis and exercise, and the transition from physiological to pathological change has been further elucidated for young Thoroughbred horses. Ongoing research focusing on the initiation and progression of OA in humans and horses will lead to early detection, treatment and management strategies to alleviate the impact of the disease and ultimately to reduce the incidence of osteoarthritis in all species.

Management of young horses at pasture with additional controlled and graded exercise may improve the ability of articular cartilage of the fetlock and other joints to withstand the rigours of athletic function. With improved knowledge of the response of tissues to conditioning it may be possible to reduce the incidence of injury in equine athletes. Research into optimising the development of these tissues to accommodate athletic performance will in turn provide insight into appropriate management, training and racing practices to reduce wastage of Thoroughbred racehorses due to lameness.

APPENDICES

Appendix 1: Viability Stain Stock Solutions

Preparation of stock solutions

1mg calcein AM and 1ml DMSO, divided into 25ul aliquots for storage.

4mg propidium iodide in 1ml of sterile water divided into 200ul aliquots for storage.

Stock solutions of fluorescent stains must be refrigerated and protected from light and calcein discarded after 6 months.

5μL of 1mM stock solution added to 1ml of DMEM for sample staining.

Appendix 2: Thresholding

The extra cellular matrix (ECM) of articular cartilage would be expected to scatter and refract light due to its significant collagen component. However background fluorescence was negligible. In pilot experiments, unstained sections of equine articular cartilage did not have significant ECM background fluorescence using the filter settings described for calcein and propidium iodide.

This may have been due to the viability stain wavelengths being above the emission frequency of equine articular cartilage collagen molecules. Which would be the case assuming that equine collagen has similar molecular structure to bovine nuchal ligament collagen with an auto-fluorescence of approximately 460nm (Swatland 1988)

Twenty images were assessed for mean background pixel intensity and a subjective threshold chosen for each one to optimally identify chondrocytes. The background pixel intensity and threshold were plotted, the line of best fit is shown below.

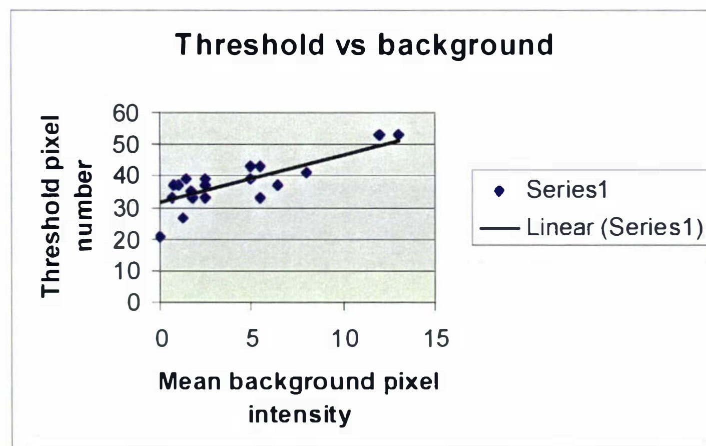


Figure A3.1: Scatter plot of mean background pixel intensity vs threshold with a line of best fit to determine appropriate threshold value for counting chondrocytes in Image-Pro Plus.

Appendix 3: Articular Cartilage Zones

Zone Criteria

Criteria used to divide the articular cartilage into zones A (combined superficial and transitional zones) and B (deep Zone) were based on cell morphology (shape and size) and arrangement of the chondrocytes irrespective of viability as determined by staining (figure A3.1).

1. A cell shape change from predominantly oval in Zone A to predominantly spherical in Zone B.
2. An increase in cell size during the transition from Zone A to Zone B.
3. A change in the predominant orientation of the cells from the long axis parallel to the articular surface or random in Zone A to the long axis vertical to the articular surface in Zone B.
4. A change in cell organisation from predominantly horizontal alignment in Zone A, to predominantly vertical columns in Zone B.
5. The notional border will be straight (if necessary those sections that do not allow a straight border will be dealt with separately).

The criteria did not all occur at the same location however one 'line of estimated best fit' was used which best followed the criteria to divide the zones.

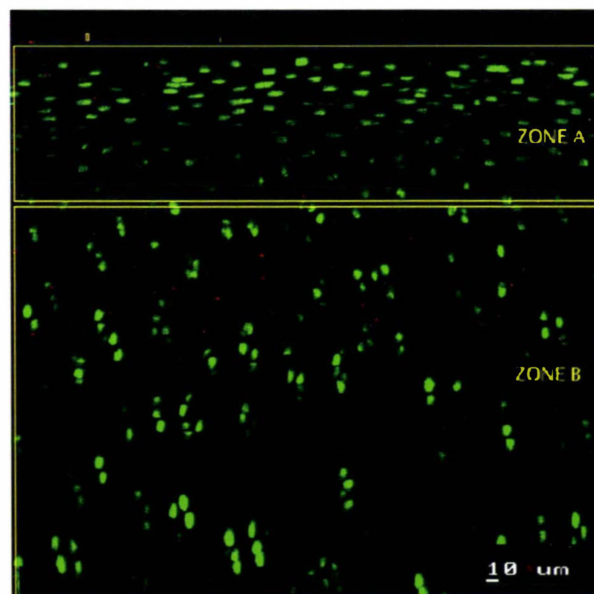


Figure A2.1: Image of articular cartilage zones for counting. Articular surface is at the top of the image, SCB at the bottom. 16x magnification.

Findings not consistent with 'normal' articular cartilage were dealt with as follows:

Abnormal findings were noted and difficulties in applying criteria recorded. For example, the articular surface was usually straight but sometimes irregular. Occasionally there were large plump circular cells present near (within 3-4 cell layers) of the articular surface even in the presence of apparently normal superficial zone. Other differences from the expected appearance of the superficial layer are the presence of small, circular, pinpoint, green staining cells giving the impression of cells with a vastly reduced amount of cytoplasm or compromised cell membrane.

Appendix 4: Histological Slide Preparation

Haematoxylin and Eosin (H&E) Preparation

Table A4.1: Haematoxylin and eosin staining procedure

Method	
1	Dewax and bring to water
	a) Two changes of xylene, 7 minutes each
	b) Absolute alcohol, until slides drain clear
	c) 70% alcohol, until slides drain clear
	d) Rinse in tapwater
2	Stain in Mayer's haemalum for 10 minutes
3	Rinse in tapwater
4	Blue in Scott's tapwater for 2 minutes.
5	Rinse in tapwater
6	Stain in 1% aqueous Eosin for 2 minutes
7	Rinse in tapwater
8	Differentiate and dehydrate in 70% alcohol followed by two changes of absolute alcohol, until slides drain clear.
9	Clear in two changes of Xylene
10	Mount with DPX
Results	
Nuclei...	Blue / black.
Other tissue components	Shades of pink to red
Strongly acidophilic structures	Bright red.

Safranin O/Fast green preparation

Table A4.2: Safranin O/Fast green staining procedure

Preparation		
Fixation	10% neutral formalin	
Process	Paraffin	
Microtomy	Cut sections at 6 micrometers	
Control Solutions	Use control specimens of articular cartilage	
Fast Green	Fast Green 1.0gm	
	Distilled water 5000.0ml[1]	
1% Acetic Acid	Glacial acetic acid	1.0ml
	Distilled Water	99.0ml
0.1% Safranin O	Safranin O	0.1gm
	Distilled Water	100.0ml

Method	
1	Decerate slides in xylene, 2 changes, 2 minutes each. Place in absolute alcohol, 2 changes, 2 minutes each; 95% alcohol, 2 changes, 2 minutes each; then rinse in distilled water.
2	Stain in working Weigert's hematoxylin for 7 minutes
3	Wash in running tap water for 10 minutes
4	Counterstain in aqueous fast green for 3 minutes
5	Rinse and agitate slides in 1% acetic acid for 15 seconds
6	Stain in 0.1% safranin O for 5 minutes
7	Dehydrate slides in 95% alcohol and absolute alcohol 3 changes each. Clear in xylene, 3 changes
8	Mount coverglass with appropriate medium (refractive index 1.48-1.56)

[1] Although the amount of distilled water used in the fast green seems excessive the formulation is correct.

Results

Nuclear chromatin	Black
Cytoplasm	Gray-green
Articular cartilage	orange-red

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