Effect of Goat milk on bone mass, morphology and biomechanics

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Abstract

Milk is a major source of dietary calcium which is essential for bone growth and maintenance, and is seen as a beneficial resource in the prevention and alleviation of osteoporotic bone loss. The objectives of this thesis were to investigate the effects of a bioactive component of goat milk, Casein phosphopeptide (CPP), and its ability to increase calcium solubility for improved calcium absorption and retention. To investigate the effect of a formulated goat milk diet as a nutritional supplement on bone growth and mineral accretion; and to investigate the effect of the long term consumption of goat milk as a nutritional supplement with or without a drug therapy (Sodium Alendronate) to determine any complementary effects on ovariectomy induced osteoporosis in the female rat. The effect of CPP on calcium bioavailability was investigated in growing rats during a period of rapid bone growth. The diets that contained 80% and 57% of goat milk protein as casein delivered increased calcium absorption compared to the diet containing 17% casein, suggesting a minimum level of casein is needed to optimise calcium absorption from goat milk. However, increased calcium absorption did not result in increased mineral retention in the femur or lumbar spine.

The next trial had two animal experiments with a total of 200 rats involved (Chapter 4 and 5); in the first experiment all 200 rats were fed either a non-milk diet, a formulated cow's milk diet, or a formulated goat milk diet from 3 weeks of age until 5 months of age. At its conclusion 60 rats were euthanized and ex vivo samples taken for analysis. The second experiment saw the remaining mature rats either ovariectomized or sham operated then grown until 10 months. The consumption of the goat milk diet increased mineral accretion during the phase of rapid bone growth beyond 'Peak bone mass' at approximately 12 weeks of age until maturity at 5 months of age. Mineral retention in the femoral shaft showed that the rats fed the goats milk diet had significantly greater quantities of mineral ($p<0.001$) compared to the not-milk group. Investigation of the marrow cavity showed that bone formation at the two cross sections examined at the femoral mid-shaft were more significant for the rats fed the goat milk diet compared to the rats fed the non-milk diet ($p<0.034$ and $p<0.007$) respectively. Ovariectomy surgery at 5½ months caused osteoporotic like conditions in bone to develop resulting in the rapid loss of bone mass in the ovariectomized rats. This saw both periosteal and

endosteal expansion resulting in larger overall marrow cavities ($p<0.0001$) in the femoral shaft and larger overall cross sectional area ($p<0.002$). Ovariectomy was also found to have an uneven effect on bone loss within the femoral shaft of ovariectomized rats (OVX), where bone at the endosteal surface had a tendency to be lost at a greater rate than the distal region compared to sham operated rats (SHAM) ($p<0.061$). This regional change showed that the SHAM rats had relatively larger bone areas in the proximal region, whereas, OVX rats had relatively larger bone areas in the distal region ($p<0.0005$).

Dual energy x-ray absorptiometry (DEXA) measurements of the lumbar spine and femur did not show any significant differences between OVX and ovariectomized alendronate groups (OVX ALD) fed either of the milk diets (Chapter 5). However, there was a potentially differing, almost opposite effect within each of the two milk diets in the bone area of the femoral shaft. The GOAT OVX rats showed a trend for larger overall mean bone areas than the GOAT OVX ALD rats ($p<0.063$), yet in contrast to this the COW OVX rats showed a trend for smaller overall mean bone areas than the COW OVX ALD rats in the femoral shaft although not significant.

The rats fed a long term diet of formulated goat milk and dosed with alendronate had a tendency to have tougher bone material per unit of bone (J/mm^2) than rats fed cow's milk and dosed with alendronate ($p<0.073$) in the femoral mid-shaft. Whereas, in the proximal femoral shaft the rats fed either of the milk diets and dosed with alendronate had tougher bone material per unit of bone (J/mm^2) than the rats fed either of the milk diets and dosed with the placebo ($p<0.05$).

Analysis of the trabecular structure of the proximal tibia showed that the rats fed goats milk and dosed with alendronate increased the prevalence of rod shaped trabeculae ($p<0.048$), increased surface volume to bone ratio ($p<0.001$), reduced the connectivity between trabeculae struts within the structure ($p<0.004$), decreased the fractal dimensions of the trabecular structure ($p<0.018$), and had thinner trabeculae ($p<0.006$) compared to the rats fed the Goat milk diet and dosed with the placebo.

In conclusion, this thesis has found that the long-term consumption of goat milk may provide some protection against ovariectomy bone loss in rats. This may be in part due to increased mineral accretion during the phase of rapid bone growth. The co-administration of goat milk and alendronate had a significant effect on the toughness of the bone material per unit area of bone in the proximal and mid-shaft of the femur, however, potentially weakened the trabecular structure of the proximal tibia.

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Abbreviations

ACP	Acepromazine
AGE's	Advanced glycation end-products or non-enzymic cross-links
α_{s2}-casein	Alpha s2 casein
α-lactalbumin	Alpha lactalbumin
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
ASTM	American society for testing and materials
BA	Bone area
β-casein	Beta casein
β-lactoglobulin	Beta lactoglobulin
BMC	Bone mineral content
BMD	Bone mineral density
BMU	Basic multicellular unit
BMPs	Bone morphogenetic proteins
BPM	Bone perimeter
BS/BV	Surface to volume ratio
BS/TV	Bone surface density
BS	Bone surface
BS/BV	Surface to volume ratio
BV	Bone volume
BV/BT	Percentage of bone volume
Ca^{2+}	Calcium ions
CO_2	Carbon dioxide
CPP	Casein phosphopeptides
CSTH	Cortical thickness
CSMI	Cross section moment of inertia
CTx	C-terminal telopeptides of type 1 collagen
CV	Coefficient of variation
DA	Degree of anisotropy
DEXA	Dual energy x-ray absorptiometry
Dpi	Dots per square inch (resolution)

ECM	Extracellular matrix proteins
EFA	Essential fatty acid
EPFM	Elastic-plastic fracture mechanics (J-integral measurement)
FD	Fractal dimension
F	Femur
G_c	Critical strain energy release rate
g	Gram
GLM	General linear model
H1-ATPase	Electrogenic proton pump H1–adenosine triphosphatase
HCl	Hydrochloric acid
ICPOES	Inductively coupled plasma optical emission spectroscopy
J	Joules
J/mm^2	Modulus of toughness or Specific energy
K_c	Critical stress intensity factor
k-casein	Kappa casein
kN	Kilonewton
LEFM	Linear-elastic fracture mechanics
LS	Lumbar spine
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor or CSF-1
mg	Milligram
mL	Millilitre
mm	Millimetre
MPa	Megapascal
N	Newton
N/mm	Extrinsic stiffness
N/mm^2	Ultimate stress
NCP	Noncollagenous proteins
ng	Nanogram
OVX	Ovariectomized rat
PBS	Phosphate buffered saline
PCA	Principal component analysis
PTH	Parathyroid hormone

QC	Quality control
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
R curves	Crack resistant curves
RUNX2	Runt-related transcription factor 2 or Cbf-alpha-1(Cbfa1)
SD	Standard deviation
SHAM	Sham-operated rat
SMI	Structure model index

Introduction

Bone is a living tissue and consists of a calcium-phosphate mineral embedded within a matrix of organic and inorganic collagen. The arrangement of the material is complex and dynamic from the micro-structure through to the macro-structure. Its primary function is structural support and mineral storage for the body. Over time the material of the bone degrades thereby reducing the quality and quantity of bone in the skeleton. This inevitable change varies in its severity depending on factors including diet, genetics and environment. One disease associated with biological aging is postmenopausal osteoporosis. It is a degenerative bone disease associated with bone loss and fracture in women for which there is no known cure. Research in this field takes one of two roles, either prevention or alleviation. Prevention methods include maximising bone mass prior to the onset of menopause so as to reduce the degree of bone loss. Alleviation methods often involve the use of drug therapies to reduce or halt bone loss. The practices of the New Zealand health system are to administer drug therapies only after the disease has been identified and osteoporotic fractures have occurred.

Skeletal mass is acquired by bone modelling and remodelling throughout life with peaks of rapid bone growth during adolescence. 'Peak bone mass', the point at which the skeleton reaches its greatest mass, is generally achieved by the end of the third decade of life. Beyond this bone mass begins to decline. Prevention research is based on maximising this peak level by optimizing bone mineral accretion and retention at periods of rapid growth and later during adulthood.

Calcium is the most abundant mineral in bone and has long been the subject of interest as a means of increasing bone mass in the young to offset postmenopausal osteoporosis. Calcium as a dietary supplement is most often consumed in milk or other diary food products. Dairy foods contain nutritional values beyond that of their individual components. Milk provides a rich source of calcium along with optimum ratios of other essential minerals and bioactive components that optimize absorption and retention of calcium in bone.

Methods for alleviating osteoporotic bone loss include Bisphosphonate drug therapies such as Alendronate. This drug is an anti-resorptive agent developed to reduce or halt bone resorption in osteoporotic bone. Another method of alleviation that has been considered is the co-administration of therapies that may have complementary effects, beyond the individual effects each therapy might offer.

There are a number of animals types used in osteoporosis research such as rats, mice, non-human primates, dogs, cats, rabbits, guinea pigs, pigs and sheep. Factors that decide what animal to use include compatibility with the human condition, cost, and availability. Rats are the most widely used animal in post-menopausal bone loss studies. The trabecular bone of rats is believed to respond in a similar manner to post-menopausal women. The rat also provides comparable results for investigations of bisphosphonate drug therapies and biomechanical testing. However, they do provide limitations, particularly when investigating changes in cortical bone, as they have limited remodelling and exaggerated periosteal growth after ovariectomy.

The principal outcome of this research was to investigate the long-term consumption of a formulated goat milk diet on mineral accretion, retention, and bone architecture in rats. The secondary outcome was to investigate any complementary effects of the formulated goat milk diet with alendronate in ovariectomized rats. The co-administration of diet and drug may improve the bones' biomechanical properties; which could allow greater amounts of energy to be absorbed before permanent damage and eventual fracture occur. In relation to these outcomes the goat milk formulated diet was also compared to a cow's milk formulated diet. This comparison was done as cow's milk is the major source of dietary calcium in the western world and is already seen as a resource in postmenopausal bone loss prevention. The co-administration of alendronate and dietary calcium from dairy foods may allow for increased mineralisation of the bone composite, which in turn may improve the biomechanical competency of the bone structure.

1. Literature Review

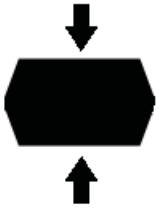
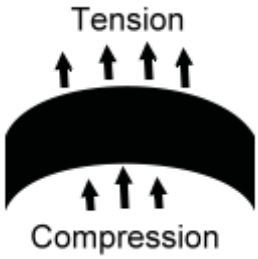
Effect of goat milk on bone mass, morphology and biomechanics.

1.1. Biomechanical Definitions

1.1.1. Stress and Strain

The mechanical behaviour of bone is often described by its structural and material properties. The material properties of bone are examined using the concepts of stress and strain (Table 1-1). Stress is defined as 'force per unit area' and is represented as either tensile, compressive or shear forces.

Table 1-1. Table of stress and strain definitions.

Stresses	Definition	Example
	Tensile stresses are forces that pull against an object.	In the femur a tension force is applied by muscle pulling on the bone (1).
	Compression stresses are forces that push on the object.	In this case the conflicting forces push on each other shortening and widening the material.
	Bending stresses involve both compression and tension on an area.	This occurs in the neck of the femur when standing or moving.
	Shear stresses are a result of forces either twisting the bone or pulling on the bone at two different points.	This can result from a sudden halt in motion or intense exercise where the inertia from body weight carried forward causes shear stresses within the tibia (2).
Strain		
As bone is stressed it is subjected to strain. Strain describes the deformation the bone undergoes. There is no 'unit of measurement' for strain, and it is often presented as a percentage of deformation (3).		

In the laboratory the relationship between stress and strain is displayed as a stress-strain diagram (Fig: 1-1). The diagram is broken into two regions - elastic and plastic. These regions explain the behaviour of the material under loading. The elastic region

generally shows a linear relationship between stress and strain as the material deforms proportionately to the applied force. This means that the bone behaves like a spring, and when the load is removed the bone will return to its original condition. This principle is referred to as "Hookes law". Although it should be pointed out that due to its composite nature, bone does not follow this rule exactly (4), however the behaviour does satisfy biomechanical testing practice (3). Once the load is applied beyond the elastic region there is permanent deformation to the material. The point at which the material leaves the elastic region and enters the plastic region is known as the 'yield point'. Beyond that the damage to the bone material is permanent, if the loading continues then eventually the material will suffer ultimate failure and fracture occurs.

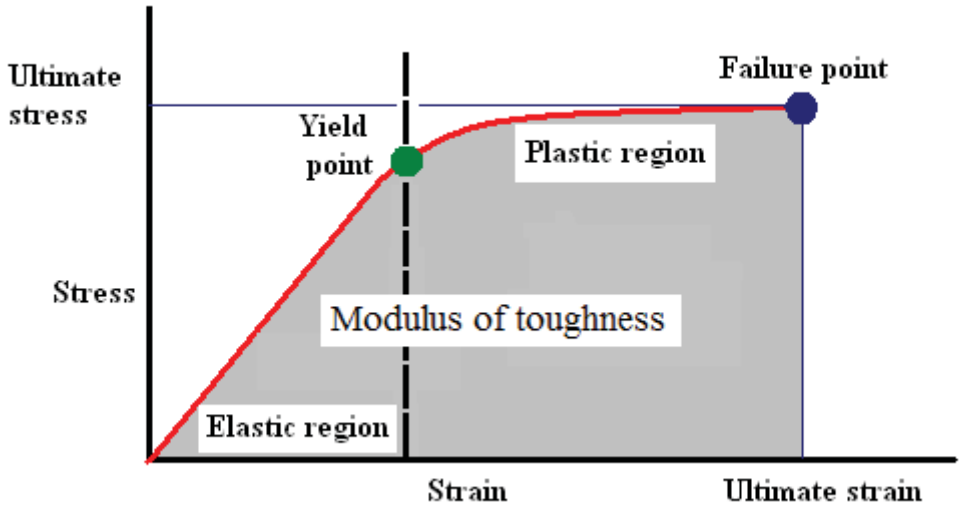


Figure 1-1. Stress-strain curve divided into elastic strain region and plastic strain region.

The stiffness of the material is determined by the slope of the linear portion of the stress-strain curve in the elastic region and the measurement of this is called "Youngs modulus" and is an intrinsic measurement.

1.1.2. Extrinsic and Intrinsic properties

Extrinsic and intrinsic properties are important concepts for describing mechanical behaviour in bone (5). The extrinsic values refer to the response the specific sample has to loading. Therefore the effect of size, shape and material composition have

direct influence on the extrinsic properties. Examples of these measurements are stiffness (N/mm), maximum force (N), maximum strain and energy (J) and show how strong the bone sample is as a complete organ (6). The intrinsic properties refer to the material that is bone. The size and the shape of the bone sample are irrelevant. Intrinsic values include Young's modulus (intrinsic stiffness), ultimate stress (N/mm^2), ultimate strain, and modulus of toughness or specific energy (J/mm^2). The intrinsic properties remove the structural elements of the bone from the equation and simply look at just the organic and inorganic components (i.e. bone material) (6).

1.1.3. Strength and toughness

Strength is the most common engineering term investigated in biomechanics. It is essentially the ability of an object to withstand a load, or the resistance of the object to yielding from the elastic region and entering the plastic deformation region where permanent damage is done (7). The stronger the object is, the more 'load' it can resist before it fractures.

Several different methods are available for measuring toughness and the most common method involves the use of notched milled specimens of bone (8-11). More traditional methods apply load to un-notched samples and measure the energy needed to fracture the bone. The un-notched sample method measures the modulus of toughness (specific energy (J/mm^2) needed to fracture the material per unit of area and represents the area under the stress-strain curve (Fig.1-1) (12). Proponents of the notched milled method argued that using un-notched samples does not take into consideration the presence of pre-existing micro-cracks in the bone composite that weaken the bone and skew results (10, 13, 14). Therefore fracture testing, using a pre-existing notch, was examined and was found to reduce the variation in findings and allowed for significant differences to be found in reduced sample sets (14, 15). The notched milled specimens use the worst case scenario where a new crack is started from a pre-existing crack (notch) (Fig. 1-2). The pre-existing crack concentrates the energy to the area and causes a concentrated crack to appear (16). However, the notch milled method does have some disadvantages of its own. The preparation of the bone samples is difficult and does introduce flaws and surface cracking in the milled areas which can pre-weaken the sample (17). Another flaw

with the notched method is the limitation of sample size, particularly if the samples are coming from bone. Studies that use small animals (i.e. rats) are unable to prepare milled notched samples because the bones are too small. Recently a tutorial was published which provided a suitable method for testing fracture toughness and crack propagation in rodents. This method tests the toughness in the largely cortical mid-shaft in the femoral long bone.

Toughness refers to the amount of energy an object can absorb before it fractures (18). Looking at the stress/strain graph, a tougher material will spend more time in the plastic region absorbing and dispersing energy before fracture, whereas a brittle material will be less able to disperse the energy through the material and fracture. Therefore tougher materials will resist the propagation of cracks leading to a fracture longer than brittle materials. Fracture toughness is one method used to investigate resistance to crack propagation, although there are no strict standards when testing biological materials. This is in part due to the inability to prepare bone samples to the correct size requirements established by the American Society for Testing and Materials (ASTM) (10). There are, however, several methods that have been adapted for bone toughness testing which include linear-elastic fracture mechanics (LEFM) and elastic-plastic fracture mechanics (EPFM). LEFM is measured using the critical stress intensity factor (K_c), the critical strain energy release rate (G_c) and crack resistant curves (R curves). LEFM theory is limited to materials that contain elastic properties except for the region around the tip of the crack which are believed to display inelastic behaviour resulting from the level of stress concentrated there (19). The inelastic behaviour forms a plastic region around the tip of the crack which increases as the load stresses increase (20). K_c (fracture toughness) describes the stress field at the tip of the existing crack. During the crack's growth the level of stresses intensifies at the tip, when the stresses exceed the materials ability to dissipate the energy (critical value) the crack will begin to grow rapidly. G_c (toughness) measures the amount of energy released during fracture along the newly forming crack per unit of surface area. The R-curve measures the toughness or resistance to crack growth while the crack is forming. This measurement is based on the theory that the bone composite material toughens around the crack and continues to toughen as the crack grows (21, 22). It has been suggested that micro-fractures occur around

the primary crack dissipating the energy of the fracture behind the advance crack, thereby reducing the crack extension force (21). Vashishth (1997) considered the K_c and G_c measurements to be of limited use in investigating bone fractures due to this toughening response in the bone material (21, 23). The R-curve is, however, limited in rat bone fracture testing due to the difficulty in recording full crack growth across small bone samples (13). LEFM measurements are considered most effective in materials that have difficulty with plastic deformation during crack growth (i.e. do not undergo post-yield deformation) (13, 19). However, there is some debate as to whether bone material fits into the definition of LEFM, in that bone has been shown to deform in the plastic region (19, 24, 25). Jan *et al* (2007) suggested elastic-plastic fracture mechanics (EPFM), or more specifically, that J-integral measurement was a better method for determining bone toughness based on the idea that it allowed for energy to be dissipated in both elastic and plastic deformation, as opposed to K_c which assumes deformation only in the elastic region (25).

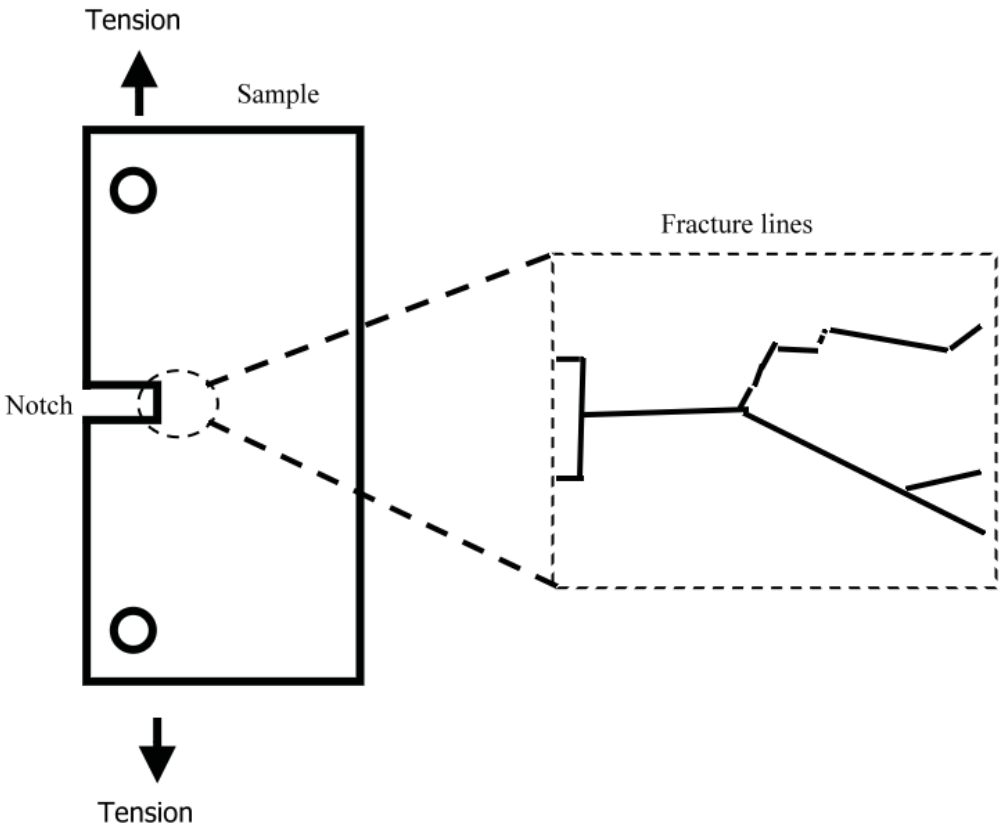


Figure 1-2. Notched milled sample under tension, as the strain increases a new crack forms as the site of the pre-existing crack.

1.2.Overview of Bone

Bone is a complex structure that provides mechanical support and organ protection, as well as providing a reservoir for essential minerals. The composite structure is made up of three main components - fibrous protein collagen (organic), mineral (inorganic), and water. The inorganic material makes up the largest portion of bone - 70% (by weight); with organic material comprising 20% - 25%. The organic portion is primarily Type I collagen with non-collagenous proteins making up 98%, and the remaining 2% are bone cells – osteoblasts, osteoclasts and osteocyte (26). The layer of cells that form on the outer surface of bone is called the periosteum and the layer of cells that form bone on the inner surface of bone is called the endosteum (Fig. 1-3).

The macrostructure of bone can be separated into two categories, cortical bone and trabecular bone. In long bones such as the femur the shaft is comprised primarily of cortical bone, while the proximal and distal regions are primarily a network of trabecular bone with a thin protective shell of cortical bone. Cortical bone is a solid structure of lamellar bone punctuated with Haversian systems (Osteon's), Volkmann canals, blood vessels and lacunae. In contrast, trabecular bone is visibly different with a network of interlacing trabeculae which has a high degree of porosity ranging from 50-90%. It is a reservoir for minerals and is the most active site for bone remodelling.

Bone is a dynamic tissue that is continually remodelled throughout life replacing old and damaged bone with new bone. This process is for the most part balanced until old age where the rate of bone loss is increased. The onset of oestrogen deficiency at menopause and senescence cause remodelling to become unbalanced resulting in increased bone resorption and decreased formation. The overall loss of bone can lead to aging bone disorders such as 'Osteoporosis' where loss leads to weakening of the composite material and can result in fracture.

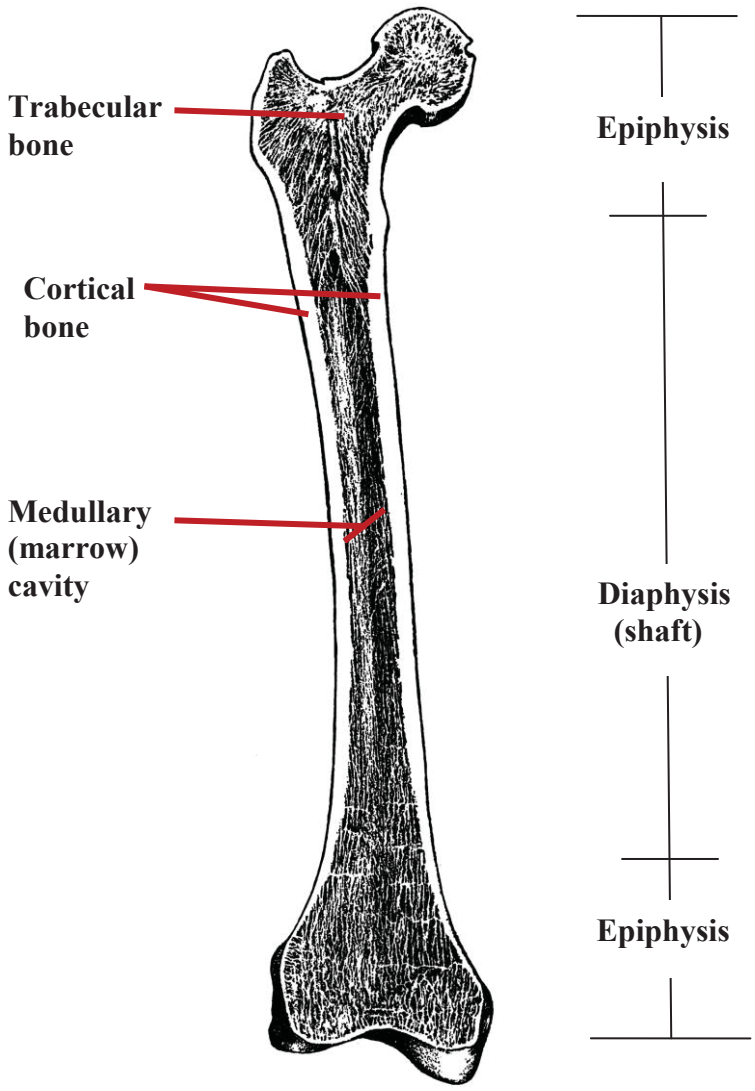


Figure 1-3. Diagram of the longitudinal cross section of the human femur, showing the epiphyseal and diaphyseal regions.

The original image is from: Blaisedell, AF. Our bodies and How We Live. Boston: Ginn &, 1904 (19). The re-mastered Image "The Right Femur sawed in Two Lengthwise" Retrieved Feburary 6, 2012, with permission for educational purposes from: http://etc.usf.edu/clipart/15400/15407/femurxsectn_15407.htm
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1.3. Bone composite

1.3.1. Mineral

The mineral found in bone contains a large number of impurities and imperfections and forms into a calcium phosphate nanocrystalline substance known as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (27). It is located in and around the collagen fibrils, is a stiff material, and provides strength to the composite structure (28). Newly formed crystals are small in size and grow as they mature. The effect of crystal size on bone strength have been investigated and it has been theorized that younger bone is stronger due in part to the mixture of different sized crystals (29). In aged bone, the weakening composite structure has been associated with increased amounts of large crystals (30). This is due to the fact that larger crystals are less capable of withstanding loading, and that the greater abundance of these crystals increased the incidence of fracture (29). At the other extreme, Gao *et al* (2003) concluded that bone material made up of small crystals could not provide adequate support, and therefore suggested that there was an optimal size for bone crystal to maximise bone strength (31).

There has been some debate over the years as to the correct shape of the hydroxyapatite crystal: it has been reported as either rod (or spindle) or plate shaped (32, 33). There is also some disagreement about how the crystal shape is formed. One theory is that the shape of the crystal is chemically driven (34), and another that octacalcium phosphate is involved in the morphological development of the crystal (35).

1.3.2. Collagen

The fibrous collagen found in bone is identified as type 1 collagen and is arranged as fibrils and each fibril is made up of three polypeptide chains in a left-handed helical arrangement. The three chains are approximately 1000 amino acids long (36) with two of the chains having the same amino acid composition and one different (2 α -1 and 1 α-2 chain). The chains are held together by a hydrogen bond linked between the peptide bond NH of Glycine and a carbonyl group in an adjacent polypeptide (37). This sequence forms a right-handed triple helix 300 nm in length and 1.5 nm in

diameter and is called a tropocollagen molecule (Fig. 1-4). The length of the tropocollagen molecule optimises the material's toughness under deformation (38). At the ends of the helix there are short telopeptides of about 11–26 amino acids per chain, which are necessary for fibril formation (39). The tropocollagen molecules form into microfibrils as they bond together with neighbouring molecules via collagen cross-linking.

Collagen cross-links generally fall into two categories; either lysine hydroxylase and lysyl oxidase mediated (enzyme cross-links) or advanced glycation end-products (AGE's) or non-enzymic cross-links. Both types have been shown to contribute to the mechanical properties of bone (40). The enzyme cross-link bonds add strength to the overall bone structure. However, when placed under tensile loading, the enzymic cross-links failed due to a loss of bonding between the mineral and the collagen that make up the links (41). Loading onto non-enzymatic collagen results in an increased transfer on load to the mineral, thereby increasing Young's modulus and decreasing the material's toughness (42, 43). AGE's cause the accumulation of cross-links in collagen leading to more fragile bone (42, 44).

The bond between the molecules is displaced by 67 nm, forming a staggered array (37). The microfibrils then aggregate to form fibrils. Collagen is arranged as fibrils and provides flexibility, high tensile strength and resistance to crack propagation to the composite of bone (24, 45). Collagen makes up 85% - 90% of the protein found in bone tissue with noncollagenous proteins (NCPs) accounting for the remaining 10% - 15% (33).

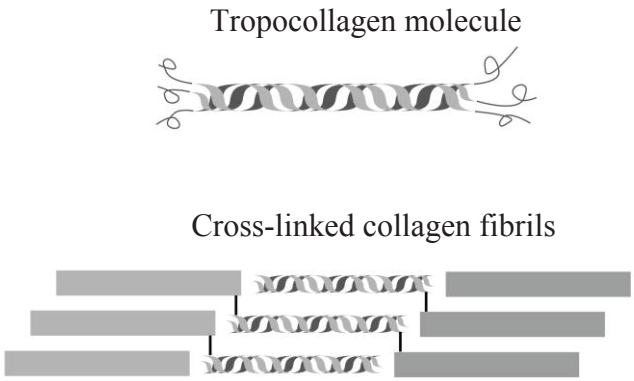

Figure 1-4. Diagram showing arrangement of the topocollagen molecule and the formation of cross linked collagen fibrils.

1.3.3. Mineralised Collagen Fibrils

Investigations of mineralised collagen are often conducted using Turkey leg tendons, and have shown mineralisation in the void spaces between the tropocollagen molecules (Intrafibrillar) (46, 47). More recent work has shown hydroxyapatite crystals growing along the surface collagen (Extrafibrillar) in the turkey leg tendon (48). The growth of mineral along the fibril has also been reported in bone collagen, with approx 75% of the mineral located on the surface (49). Both intrafibrillar and extrafibrillar mineralisation are important for mechanical strength. However, it has been suggested that intrafibrillar mineralisation plays the dominant role in the mechanical competence of the bone tissue (50). It has also been theorized that the orientation of the hydroxyapatite crystal may influence the mechanical strength of bone, although there is some debate over its correct orientation. Some researchers have suggested that the mineral is orientated in the same direction as the collagen fibrils; whereas others argue that the orientation of the mineral in relation to the fibril is dependent on its location, either within the fibril, or on the surface of the fibrils (49, 51). Part of this argument was based around the idea that the tendon was not a practical comparison for bone collagen mineralisation.

The toughness of bone can be determined by how much energy it can absorb and plastically deform before it fractures. At the micro-structural level fibrils may assist with enhancing energy dissipation by the presence of bonds known as ‘Sacrificial

bonds'. Fantner *et al* (2005) examined this theory in bone undergoing fracture and found the presence of a non-fibrillar organic matrix that bonded the mineralised fibrils together (52). While exposing the fibrils to mechanical force, these sacrificial bonds failed under stresses less significant than was needed to permanently damage the fibrils. The failing bonds lengthen the fibrils allowing the energy to dissipate through the material resulting in a tougher material (53). They also noted that the bonds reformed after they were broken, thereby reinforcing the toughness of the composite material (52).

Mineralised collagen fibrils may also assist toughness by creating localised regions to contain stress related micro-damage. These toughened regions of material form around pockets of damaged bone and allow micro-cracks to grow in order to dissipate the energy within the localised region, while protecting the entire structure from the formation of macro-cracks (54). The formation of micro-cracks in cortical bone are believed to toughen the overall macro structure (10, 23).

In bone the ratio of hydroxyapatite crystal to collagen fibres in the composite reflects the stiffness of the material. Stiffness is another measure of mechanical behaviour, and reflects the integrity of the bone (6). Bone that is highly mineralised will be stiffer than bone that has a greater proportion of collagen (55). A reason for the variation in bone stiffness can be contributed to its function (56). Deer antlers are used as a fighting tool and are required to withstand significant impacts without fracturing; therefore, having a lower percentage of mineral increases the resistance of the material to crack formation (57). The more mineralised the bone the easier cracks will travel through the material. When the cracks hit organic components (i.e. collagen) the flow of the crack is disrupted (56). At the other end of the scale, whale bulla (inner ear bone) has a high level of mineralisation and material stiffness; this is necessary for the auditory function (55).

1.3.4. Collagen Fibril Array

The fibril array is formed from aggregated fibrils, with each array then forming a collagen fibre. The fibril array is an anisotropic material and it is strongest when

forces are acting in the direction of the fibrils' orientation. The fibres then orientate themselves into woven, parallel, or plywood configurations. Woven bone is found in human foetuses where it is present for the first few years of life until it is gradually replaced by lamellar bone. After a fracture in mature bone, woven bone is the initial bone laid down in the repairing of that damaged mature bone. The woven bone composite is comprised of fine collagen fibres, with a high degree of mineralisation. The nature of its biomechanical properties has not well understood but is considered to be less structurally supportive than lamellar bone due to the random orientation of the collagen fibrils (58, 59). Parallel-fibered bone, as the name suggests, has a more parallel structure to its fibril organisation than woven bone, and is orientated parallel to the periosteal surface (60). The most structured bundles of collagen fibres are organised into lamellar bone which has the appearance of a plywood-like structure. The different layers (lamellae) that make up the lamellar structure have different thicknesses, where a thin lamellae is followed by a thicker one (36). These layers are offset by approximately 30° increments from 0° to 120° (61). The orientation of these lamellae have been reported to be able to rotate by as much as 90°, allowing for greater resistance to multiple directional forces (62). The plywood model was introduced by Gebhardt in 1906 and is viewed by many as an accepted model for lamellar bone structure, however not all agree with this explanation. Marotti (1993) suggested an alternative model where the different layers are made up of an arrangement of densely packed collagen, followed by loosely packed collagen lamellae (59).

1.3.5. Non-collagenous proteins

There are two major classes of noncollagenous proteins (NCP) found in the extracellular matrix - multiadhesive matrix proteins and proteoglycans (37). Of these two classes the main NCPs are osteocalcin, osteonectin, integrins, growth factors and cytokines. Osteocalcin is produced by osteoblasts and is often used as a bone marker for the assessment of bone formation. The primary function of multi-adhesive matrix proteins is to attach cells to the extracellular matrix; proteoglycan proteins have a high level of hydration which allows them to help provide protection by cushioning cells.

Osteonectin is an extracellular matrix glycoprotein that is secreted by osteoblast cells during bone formation. It initiates mineralization and promotes the formation of apatite crystal.

1.3.6. Water

Water keeps the composite bone hydrated and represents approximately 25% of the bone's total volume (63). It can be found in the void spaces in bone as well as the extracellular matrix (64). Biomechanical testing of *ex vivo* bone has shown significant differences in strength and toughness between dried and hydrated samples (36, 65). Dried samples have increased stiffness but a reduction in energy to fracture (3). Townsend *et al* (1975) noted that single hydrated trabeculae displayed ductile behaviour, whereas single dried trabeculae had a more brittle response to compression (66).

1.3.7. Haversian Bone (secondary Osteons)

Haversian systems make up 5% - 8% of lamellar bone volume and are formed by osteoclasts as the bone undergoes remodelling. They develop longitudinally through the lamellar bone and are interconnected by Volkmann's canals. These systems are cylindrical in shape and hollow in the middle to accommodate blood vessels. The presence of these systems is known to compromise the compression strength of long bones subjected to bending forces (33). Under such strain Ebacher *et al* (2007) observed micro-cracking within the Haversian systems in human tibia (67). They concluded that Haversian systems influenced both bone deformation and fracture, and that these findings were particularly relevant to the elderly (67). In contrast, bone cracking caused by tensile forces from bending, was less sensitive to the presence of Haversian systems (68). These varying responses beg the question as to whether there are regional variations to the locations and densities of Haversian systems. Using an electron microscope Pazzaglia *et al* (2009) showed regional differences in Haversian system morphology between the mid-shaft and distal femur, as well as higher densities at the endosteal surface, compared to the periosteal surface in the mid-shaft in mature rabbits (69).

1.4. Bone cells

The three bone cell types: osteoblasts, osteoclasts and osteocytes work to lay down new bone (modelling) or to reabsorb and replace old bone (remodelling). Modelling occurs in children and adolescents increasing the size and density of bone. During modelling, bone formation is not immediately preceded by resorption; in these instances the activity of osteoblasts and osteoclasts work independently of the other. As the bone reaches maturity the incidence of modelling is reduced. In adult bone, modelling events have been reported in response to mechanical loading and fracture repair (70). Remodelling occurs throughout both cortical and trabecular bone. On average approximately 2% - 5% of cortical bone is remodelled each year. Trabecular bone remodels at a greater rate, approx 25% faster, due to its larger surface area (71). Overall approximately 10% of bone is remodelled every year and continues at that rate throughout life (72).

1.4.1. Osteoblasts

Osteoblasts are mononucleated cells that differentiate from mesenchymal stem cells in the bone marrow (73, 74). There are several stages of osteoblast differentiation; these are proliferation, maturation and termination. Each stage is controlled by growth factors which determine the cells' function including members of the hedgehog gene family, bone morphogenetic proteins (BMPs), transforming growth factor *beta* (TGF-β), and canonical Wnt (Wingless-type). These growth factors are signalling pathways that communicate to transcription factors for osteoblast differentiation.

RUNX2 (also known as Cbfa1) and Osterix are two transcription factors needed for osteoblast differentiation (75). Mice deficient in RUNX2 have a complete lack of ossification within the skeleton (76). RUNX2 was first identified by Nakashima *et al* (2002), their research showed mice deficient in Osterix had a complete lack of bone, from this they offered the theory that RUNX2 regulated the role of Osterix (77). They also noted that the cells expressed RUNX2, and therefore suggested that Osterix operated downstream from RUNX2 in osteoblast differentiation. This was later demonstrated by Nashio *et al* (2006), where Osterix transcription was both stimulated and responsive to RUNX2 (78).

Mature osteoblasts are found at the periosteum and endosteum surfaces. They form a layer of bone matrix by secreting extracellular matrix proteins (ECM). These proteins contain largely type I collagenous proteins, noncollagenous proteins, BMPs, and growth factors (79). As the matrix forms and calcifies some osteoblast cells are trapped and become osteocytes. The termination phase of osteoblast cells occurs once mature cells become osteocytes or bone lining cells. Bone lining cells are osteoblasts that are not actively involved in formation; they appear at remodelling sites and have a flattened appearance.

1.4.2. Osteocytes

The trapped osteocytes occupy cavity spaces known as lacunae and form connections with other osteocytes and with bone-lining cells through dendritic processes (80). These processes are housed in small canals called canaliculi. They are the most abundant cell in mature bone and are believed to act as mechanosensors for the control of bone formation and remodelling (81). This communication network may respond to mechanical stimulation from loading on the bone tissue. The loading is transmitted though the solid and fluid materials stimulating bone cell activity (82, 83). In recent years research has found that osteocytes may also respond to direct biochemical stimulation for the control of remodelling.

1.4.3. Osteoclasts

Osteoclasts are multinucleated cells that differentiate from the bone marrow macrophage (84). Their differentiation from osteoclast precursors to mature multinucleated cells requires two essential cytokines -'receptor activator of nuclear factor (NF)-κβ ligand' (RANKL), and macrophage-colony stimulating factor (M-CSF) or CSF-1. M-CSF is necessary for the proliferation, differentiation and survival of osteoclast precursors (74, 85). RANKL is a ligand for 'receptor activator of nuclear factor (NF)-κβ' (RANK) and is secreted by osteoblasts. The interaction between osteoclast precursors that have the RANK receptor, and the osteoblast bone lining cells expressing RANKL, activates mature osteoclast cell formation. Mature osteoclasts are attached to calcified bone tissue and within lacunae, and the function

of these cells is bone resorption. Once resorption of a site is complete the osteoclast enters its final phase and cell death occurs (apoptosis).

1.4.4. Immune cells

T-cells, B-cells and Osteomacs are some of the immune cells that influence remodelling. T-cells and B-cells are lymphocytes that assist in the regulation of bone resorption by expressing osteoclastogenic cytokines such as RANKL leading to mature osteoclast formation (86). Mice deficient in either B-cells or T-cells that have undergone ovariectomy surgery do not have the increased resorption or bone loss associated with the surgery (87). Similarly the number of active T-cells in healthy postmenopausal women is lower compared to osteoporotic postmenopausal women (88). Osteomacs are resident tissue macrophages that have recently been indentified in or on bone cells in endosteal and periosteal bone tissues (89, 90). *In vivo* work in mice has reported osteomacs assist in the regulation of osteoblast function at bone modelling sites (89). *In vitro* work with culture prepared from human cadavers found that there was reduction in osteoblast mineralization in cultures that lacked osteomacs macrophages (89). The researchers suggested that osteomacs have an essential role in bone homeostasis via regulation of osteoblast function (89).

1.5.Remodelling

Remodelling involves the mutual resorption of old bone by osteoclasts and the laying down of new bone by osteoblasts. These cells work in unison along with osteocyte and bone line cells in a temporary arrangement referred to as the Basic Multicellular Unit (BMU). This arrangement is contained in its own environment under a canopy of bone lining cells (91, 92), which provides a balanced removal and replacement regime to the degree that the rate of change in overall bone mass is marginal (71). However, this exchange is only truly balanced for a short period of time during adulthood between the cessation of bone growth, and the inevitable loss of bone accompanying ageing.

The arrangement of BMU's is different in cortical bone compared to trabecular bone. In cortical bone the BMU is organised in a cone shape with osteoclasts on the leading edge burrowing through the composite material. These are followed by reversal cells

which prepare the bone surface for formation. Finally the osteoblasts occupying the tail of the BMU lay down new bone. In trabecular bone the BMU's move along the trabecular surface removing and laying down new bone in the pancake like pockets created in the resorption process.

The remodelling sequence is described in six phases, starting with cell activation at the resting surface and ending with the re-establishment of the resting surface (93).

Once activated the pre-osteoclasts mature and are attracted to the bone surface. Bone lining cells remove non-mineralised bone matrix which allows mature osteoclasts to attach to the resorption site. The mature osteoclast establishes a compartment between the cell and the bone surface and contact is identified by the ruffled border of the osteoclasts plasma membrane. The osteoclast secretes enzymes and biochemical material across the plasma membrane into the closed space. The acidification of this area dissolves the bone crystal and demineralises the bone. These secretions are mediated by an electrogenic proton pump H1–adenosine triphosphatase (H1-ATPase) and chloride channels (94). The secreted enzymes digest the organic components and the remaining degraded bone is then transported across the cell and expelled into the extracellular space (95). Once resorption has occurred there is a short time of reversal in which the cement line is formed. This is followed by osteoblasts forming extracellular bone matrix (Osteoid). Formation continues and the new matrix becomes mineralised with calcium and phosphorus. The process ends when mineralisation is complete and a new layer of non-mineralised bone matrix is laid down, covered by a layer of bone lining cells. Once the process has ended the area is once again a resting surface.

1.5.1. Initiation of bone remodelling

The remodelling sequence is initiated by activation at the resting surface by mechanical and biochemical stimulation (96, 97). The mechanical strain on the bone is detected by the osteocyte network via direct load transfer, or through changes in fluid pressure within the extracellular matrix (98-102). Osteocytes are extremely sensitive to the fluid shear stresses caused by the fluid flow within the canalicalur network (103), and respond by releasing messengers such as prostaglandins and nitric oxide (104, 105). The resulting signals are thought to move through the network via

gap junctions towards the bone lining cells on the surface (106, 107). Once activated the bone lining cells secrete chemo tactic cytokines - Monocyte chemoattractant protein-1 (MCP-1), which attracts osteoclast precursors (108). The bone lining cells then remove the non-mineralised matrix on the bone surface, which in turn allows the multinucleated osteoclasts to attach to the resorption site.

The magnitude of strain detected by the osteocyte network determines the level of modelling or remodelling activity (97), and a minimum amount of strain is required for bone maintenance (109). Therefore, if strain is sufficiently increased above maintenance then bone modelling is stimulated and bone mass increases. This kind of scenario occurs in athletes who repeatedly subject their bodies to strenuous activities resulting in bone growth at the periosteal surface of long bones (110), or rat limbs that are repeatedly subjected to cyclic loading (111).

Work conducted in the 1980's introduced the idea that remodelling occurs not only to replace old bone but also to replace bone damaged with micro-cracks caused by fatigue (112). Martin (2002) explored this theory using mathematical equations to further examine whether remodelling was driven by micro-damage in the bone, or more closely followed the traditional belief that remodelling was driven by metabolic requirements (113). His results indicated that BMU's appeared in close spatial proximity to one another. However he did point out that there was insufficient data available to test his model (113). It has been theorised that this process is triggered by damage, death or isolation of the osteocyte at the location of the micro-damage causing the signal to be either severed or suppressed (114). Regardless, this would have the potential to prevent the osteocyte processes from suppressing osteoclast activation and allowing remodelling to occur (115). Osteocyte apoptosis is considered necessary for the initiation of remodelling, and signals either or both osteoclast and osteoblast activity (116). Signalling occurs when the normal levels of growth factor TGF-β secreted from healthy osteocytes are lowered in the damaged cells (117, 118).

The other extreme response to strain occurs with astronauts and people who are incapacitated for long periods of time. In these cases individuals are subjected to

bone loss due to the reduced strain stimulation of zero gravity or inactivity from prolonged bed rest (119, 120). Investigations on restricted use of limbs in young adult dogs found reduced growth, particularly at the periosteal surface. In older dogs the restricted limb use increased resorption at the endosteal surface, increased rapid remodelling and porosity in cortical bone (121, 122). Immobilization of a hind limb in growing rats decreased bone mineral content and resulted in the loss of trabeculae (123). In a weightless environment, the reduced stress on bone increased remodelling at the endosteal surface and trabecular bone (124).

1.5.2. Regulation of bone remodelling

There are a number of systemic regulators of bone; these include growth hormones, oestrogen, calcitonin, PTH and 1, 25-(OH) 2-vitamin D3. The growth hormones (GH)/IGF-1 and IGF-2 promote bone growth especially during endochondreal formation. They have been shown to increase bone formation by direct interaction with growth hormone receptors on osteoblasts, and through an induction of endocrine and autocrine/paracrine IGF-I (125). Glucocorticoids have a negative effect on bone resulting in a decrease in formation. The exact cause of this effect is unknown but it has been suggested that glucocorticoids sensitize osteoblasts to regulators of remodelling (126). Recently oestrogen was found to maintain the levels of osteoblasts and osteoclasts by inducing osteoclast apoptosis via signalling through the Fas ligand pathway (127).

PTH (Parathyroid hormone) regulates the calcium balance within the body (calcium homeostasis) and is a systemic regulator of remodelling. It is secreted from the parathyroid gland in response to reduced calcium serum levels. This in turn stimulates resorption resulting in the release of stored calcium from the bone reservoir. Resorption is initiated in scenarios where continual secretion of PTH occurs. However when PTH is administered intermittently, bone formation in both rats and humans can be stimulated (128, 129). The process of PTH activating resorption follows the process of PTH binding to its receptor, a seven transmembrane G-protein-coupled receptor on osteoblastic cells. This action in turn increases production of the receptor activator RANKL and M-CSF leading to osteoclast differentiation, activation and resorption. O'Brian *et al* (2008) reported that activating

the PTH receptor in osteocytes increased bone mass and remodelling in transgenic mice (130). PTH also stimulates 1, 25-(OH) 2- D3vitamin production which in turn stimulates calcium absorption from other organs such as the gut and kidneys. Calcitonin also works to maintain calcium homeostasis in the body and affects bone resorption by inhibiting the resorption of extracellular calcium ions (Ca^{2+}) (131).

1.6.Structural design of bone

In the 19th century Julius Wolff and Wilheim Roux formulated a theory of bone remodelling in relation to biomechanics, known as "Wolff's law". This law states "The principle that every change in the form and the function of a bone or in the function of the bone alone, leads to changes in its internal architecture and in its external form" (132). This theory has been used to offer, in part, an explanation for the architecture of bone and that mechanical requirements placed on the bone will determine how the bone material is laid down and where.

Long bones such as the femur are subjected to the forces of gravity and the weight of the body's mass and needs to be a compromise between weight and mechanical competency. The biomechanical method used to examine the effect of loading on the femur shaft is borrowed from the engineering principles used to explain the behaviour of beam structures (133). In engineering terms, to build a cylindrical structure (i.e. a beam) to maximise strength, a hollow cylinder would be used with an external diameter as wide as possible. The limitation placed on the cylinders' external diameter given a set amount of material is related to the minimum thickness the cylinder wall can be before it buckles (33). As stated by Currey (2003) "weight for weight, a hollow cylinder is stiffer, and to a lesser extent, stronger in bending than a solid cylinder" (134). Under axial loading (i.e. standing) the shaft of the femur is subject to tension, compression and shear forces. The load directed down the long axis of the femur is conducted through the walls of the bone cylinder that form the shaft. These forces are directed outwards away from the neutral axis (marrow cavity) and are greatest at the outer perimeter of the cylinder (periosteum of the femoral shaft). Therefore, bone formation at the periosteal surface will in theory increase the bending strength of the femur. This phenomenon has been reported where even small

increases in cross sectional area via periosteal expansion have added significantly to the long bones bending strength (135, 136). This expansion increases the second moment of inertia, and the resistance to bending is measured using 'Cross Sectional Moment of Inertia' (CSMI). Geometric properties including CSMI have predicted up to 70% - 80% of bone biomechanical competence (137). However, they can be an even stronger predictor when used in conjunction with bone mineral measurements (136-140). Augat *et al*(1996) found that CSMI and 'Bone mineral density' (BMD) measured together best predicted biomechanical competence at the distal radius, femoral neck and lumbar spine of cadavers using a peripheral computed tomography bone scanner (pQCT) (136).

There is some debate as to whether bending is the correct description for the type of stress that the femur diaphysis is subjected to under axial loading (141). Biomechanical testing of the femur is often conducted with bone samples that have been stripped of soft tissue. In humans, *in vivo* testing that takes into consideration muscle activity around the femur in response to loading has found that compression is the primary force expressed on the diaphysis of the femur with bending being less significant (142). During locomotion, however, bending forces have been identified in the mid-shafts of humans and horses *in vivo* (142, 143). Strain gauges recorded compression strain in the anterior cortical bone of the mid-shaft and tension strain in the posterior cortical region of the horses radius (143). Further, in humans the bending forces seen during motion have been reported to alternate in the femur as the gait progresses rather than bend in only one direction (142).

Cortical bone is weaker under tension compared to compression. When subjected to axial bending a femur will fracture at the area under tensile stress rather than the area subjected to the compression (33). The bone composite may compensate for this by altering the architecture of the structure to accommodate the stresses applied to different regions. The regions of horse long bones that are subjected to tensile stress tend to have collagen fibres arranged in a longitudinal direction, whereas, areas of repeated compression tend to have collagen arrangements that are primarily directed in a transverse direction (143). Similarly, Haversian systems are arranged in orientations that provide the best structural strength to a particular region. Haversian

systems are strongest when arranged transverse to the direction of the load in regions that are frequently subjected to compression (144). In areas where tensile stresses are common the Haversian systems are orientated in the longitudinal direction (145).

Bone is an anisotropic material, which means that it will respond differently to loading depending on which direction the force is applied. Under longitudinal compression human cortical bone has an ultimate strength of 193 MPa, yet if compression is applied perpendicular to the direction of the collagen fibrils (side on), then cortical bone strength is 133 MPa (146). Trabecular bone has a complex structure and the orientation of the trabeculae significantly influence the strength of the material (147). Determining the degree of anisotropy in trabecular bone was traditionally done using two-dimensional imaging but has more recently been done with three-dimensional imaging using a μCT scanner (148). The three-dimensional process involves looking at the arrangement of the bone area and empty spaces along any line of structural orientation. When a line is drawn through an isotropic structure it would be expected to show a comparable number of intersections between that line and the bone under any orientation; whereas, the number of intersections in an anisotropic structure would depend on that structures orientation. Post-menopausal bone becomes more isotropic as bone loss reduces the complexity of the structure (149).

The complexity of a structure's architecture, or how well it fills the space within the structure, can in part determine its strength. The term that describes this is 'Fractal geometry', and objects that contain fractal dimensions (FD) tend to have complex repeating architectural patterns. These patterns have a similar visual appearance when viewing the whole object or a smaller segment of that object. Fractal geometry has been successfully used to describe changes that occur in trabecular bone as a result of bone cell activity (150). Fazzalari & Parkinson (1996) took biopsy samples from 25 human subjects between the age of 39 to 90, and successfully used fractal analysis to detect changes in the complex structure of trabecular bone resulting from remodelling activity which altered trabecular number, spacing and thickness (150). This method has also detected the change in iliac trabecular bone architecture where elderly osteoporosis patients had significantly lower FD than healthy elderly subjects (151).

The proximal and distal regions of the femur are filled with the trabecular network which allows for the distribution of stresses across a greater surface area of bone. The trabeculae that make up this network are aligned along these stress vectors providing greater strength to the overall structure (74), and is known as stress trajectory theory (152). This is an area of contention amongst scientists, as it has yet to be determined if biomechanical stresses are solely responsible for this arrangement, or if growth patterns also determine the long bones architecture (119). Biewener *et al* (1996) investigated trabecular architecture of calcaneus bone in 'Potoroos' (a small marsupial), after the Achilles tendon was surgically removed to prevent load bearing on the region (153). They observed that while there was a generalized loss of mineral from the trabecular region the fundamental structural alignment was not affected. They theorized that trabecular alignment was formed in response to loading patterns during growth, and these patterns could not be altered in mature adult bone regardless of changes in the functional loading of the bone (153). Altered loading patterns have realigned the trabecular structure of the proximal tibia in humans and dogs suffering from osteoarthritis (154, 155). Barak *et al* (2011) also noted that trabecular bone adjusted and realigned its structure in relation to changes in peak loading on joints in the hind limbs of sheep subjected to trotting exercise at different inclines for a month (156). Whatever the case, it is generally accepted that the trabeculae tend to align themselves along paths of maximum stress. In the lumbar spine, femur and proximal tibia the mechanical competence of the trabecular bone material is strongest and stiffest when loading is directed along the trabecular alignment.

1.7. Osteoporosis

Osteoporosis is characterized by a reduction in bone density and in bone strength leading to pathological fracture. It affects upwards of 50% of New Zealand's female population over the age of 60. Post menopausal bone loss falls into two categories where there is an initial rapid loss of bone and then the loss becomes more gradual. Initially the bone is lost at a rate approximate to 10% per decade until the approximate age of 75 then this is reduced to approximately 3%. The loss is more significant at trabecular sites with 20 – 30% of bone lost compared to cortical regions which suffer

5 – 10 % bone loss (157). Subsequently it has been suggested that beyond the age of 50 years the risk of fracture in women suffering from osteoporosis is as much as 40% (158). There are currently two approaches being investigated to combat this condition, prevention by increasing 'Peak bone mass', and therapies to alleviate post menopausal bone loss. 'Peak bone mass' represents the accumulation of bone mass prior to the inevitable bone loss that accompanies ageing, i.e. onset of menopause. Prevention studies in humans and animals suggest that monopolizing this bone mass to its full genetic potential assists in reducing the level of loss (159-164). Bone growth throughout childhood and adolescence reaches approximately 90-95% of its peak mass by the end of the 2nd decade. 'Peak Bone Mass' is reached between the 2nd and 3rd decade of life, although different skeletal regions achieve peak bone mass at different times (165) (Fig. 1-5). Exercise, a balanced diet, and sufficient dietary intake of calcium and other minerals throughout the 1st and 2nd decade were linked to increased 'Peak bone mass' and reduced osteoporotic fractures in the elderly, compared to women who are inactive and have a 'poor' diet (166-168). Recker *et al* (1992) also found that increased calcium intake and a more active lifestyle in woman could provide small yet significant gains in bone mass during the 3rd decade (167).

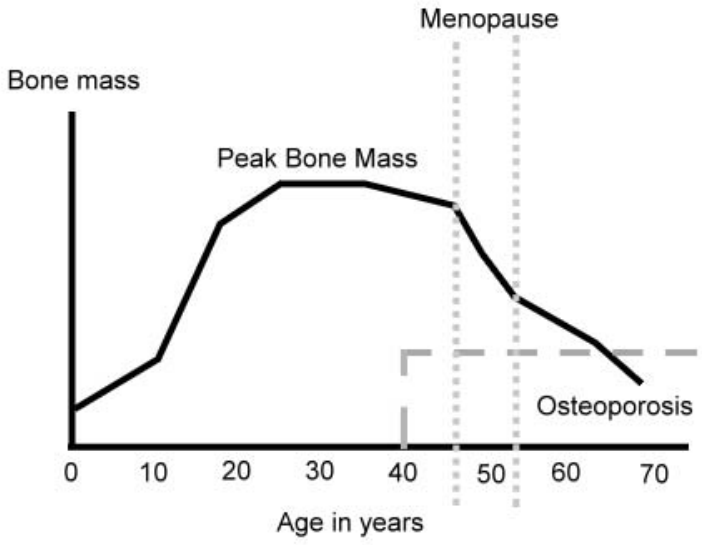

Figure 1-5. Bone mass lifecycle for Women, highlighting 'Peak bone mass' and the decline of bone with aging.

1.7.1. Misregulation of remodelling

Bone loss brought on by the onset of menopause results from the uneven turnover of bone coupled with an increase in remodelling. The regulatory processes that govern remodelling are disrupted by biological aging which can prevent the formation of osteoblast precursors (169). Senescence also causes an oestrogen deficiency which unbalances the BMU by increasing the lifespan of osteoclasts yet reducing the life of osteoblasts (170). When balanced, oestrogen regulation includes the suppression of inflammatory cytokines such as interleukin (IL)-1, interlieukin (IL)-6, and tumour necrosis factor (TNF) (171). Oestrogen deficiency allows these cytokines to activate osteoclast maturation via RANKL, thereby promoting bone resorption (172). It has also been theorised that defective osteocyte function could assist in the onset of osteoporosis (78).

The size and presence of larger sized hydroxyapatite crystals increases with age, resulting in increasingly brittle bone material. The onset of osteoporosis further increases the quantity of larger sized crystals (29, 173). Osteoporosis does not alter the orientation of the mineral within the composite (71). Femur samples were collected from skeletal remains of similarly aged humans in a population known to suffer a high incidence of osteoporotic vertebral fractures (174). The bones that had osteoporosis were characterised by larger crystal sizes compared to bone samples taken without the disease (174).

Currently the actual amounts of enzymatic collagen cross-links and AGE's cross-links in reference to bone quality in human osteoporotic bone is unknown (40). There is evidence that increasing age and the onset of osteoporosis reduced the quality of bone collagen even though the density of collagen did not decline (175-177). The reduced quality of bone was due to a reduction in mineralization, a lower number of enzymatic cross-links, and excessive amounts of pentosidine (a biomarker for AGE's) (177). An increase in AGE's cross-links is said to decline the ability of the bone to dissipate energy resulting in fracture (54).

The structural effect of uneven bone turnover causes the thinning of the cortical wall in the shaft of long bones. This type of architectural change results when bone

formation at the endosteal surface ceases, coupled with continued erosion from the endosteum as well as periosteal apposition. A possible cause for the periosteal expansion might be in response to reduced structural strength brought on by the thinning cortical wall (178). In rats ovariectomy induced osteoporosis causes similar bone responses as postmenopausal osteoporosis in women. However, the degree of periosteal apposition is more exaggerated in rats (179, 180).

The development of two and three dimensional imaging methods such as μCT scanners, has allowed for detailed investigations of the structural changes that occur in the trabecular regions of osteoporotic bone. Osteoporosis alters the degree of connectivity, number, thickness, shape and structure of trabeculae; all of which can alter the biomechanical behaviour of the material resulting in a weaker structure (181-185). The individual trabeculae that make up the structure have been described as either plate-like or a rod-like shape; with a higher degree of plate shaped trabeculae reflecting a more connected structure. Reports in both human and rat studies have indicated that osteoporosis causes a transition from plate-like shape trabeculae to rod-like shapes (149, 186). This shift also reflects the altered surface structures away from the more connected concave surfaces to the less connected convex surfaces (181). In women the rapid bone loss represents the complete removal of entire trabeculae leaving the structure more porous (182). To date there is no known treatment that replaces the lost trabeculae and repairs the overall structure to pre-osteoporotic conditions (187). There is some evidence in rats that existing trabeculae do thicken, possibly as a means of support to the weakened structure (188). Weinstein & Majumdar (1994) used Fractal analysis to assist in the prediction of vertebral bone fragility in elderly osteoporotic women (183). When comparing the patterns of branching trabeculae within the vertebral structures, women who had a history of compression fractures had lower FD patterns (i.e. less trabeculae branching) (183).

1.7.2. Effects of Bisphosphonates on Osteoporotic bone

Bisphosphonates such as alendronate are anti-resorptive therapies designed to arrest resorption in osteoporotic bone. Bisphosphonates have a structure containing two phosphonate groups attached to one carbon atom ‘P-C-P’, which binds to the

hydroxyapatite crystal in bone (Fig. 1-6). Primarily this binding occurs at remodelling sites preventing the resorption of crystals by mature osteoclast cells (189).

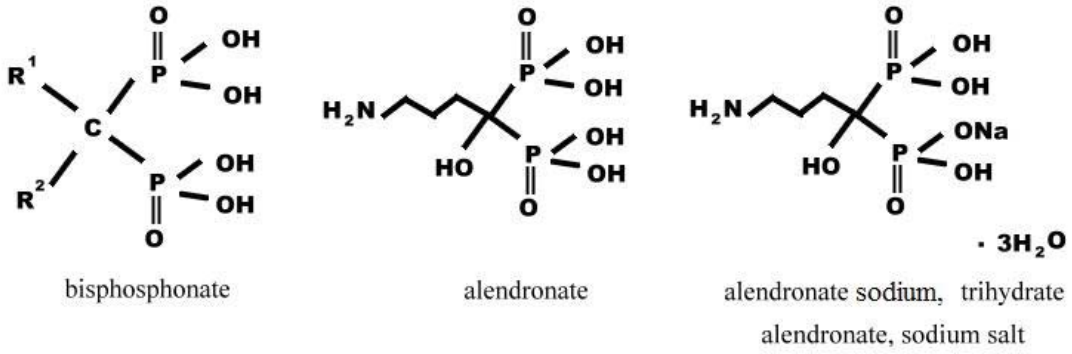

Figure 1-6. Structure of Bisphosphonate, alendronate and the chemical name (alendronate, sodium salt).

Alendronate is also a strong inhibitor of osteoclast action. Bisphosphonates are released from the acidified bone surface during the resorption phase, and are then internalised into the osteoclast cell. Coxon *et al* (2008) was able to show that osteoclast cells were able to absorb significant amounts of bisphosphonates *in vitro*, although they were unable to determine how exactly the process of internalisation occurred (190). There are several theories as to what effect this has on the osteoclast cell. One theory suggests that some bisphosphonates are associated with cytotoxic effects on mature osteoclast cells during their resorption cycle (191, 192). The toxic effects may be triggered by high levels of the drug being administered (193, 194); in cases where lower concentrations of bisphosphonates were used, a toxic reaction was not found (195). During these toxic reactions the level of resorption is decreased, possibly due to a reduction in the number and size of resorption pits at the remodelling sites (191). An alternative theory suggests that when osteoclasts attempt to resorb bone mineral bound to alendronate, they experience cell death due to altered cell morphology (194, 196-198). The most notable morphological change seen in osteoclasts exposed to bisphosphonates, is the loss of the plasma membranes ruffled border. The lack of this border prevents the cell from adhering to the bone surface reducing the level of resorption (194, 199, 200).

Bisphosphonates such as alendronate do not alter the ratio of mineral and collagen in bone (45), yet there is a significant increase in both mineralisation (59) and the AGE's 'Pentosidine' collagen cross-links (43). AGE's cross-links increase the level of strain on mineral diverting it away from the collagen, resulting in a decrease in the toughness of the bone material (42, 43, 176, 201, 202). Mature female dogs given alendronate at a dose rate of 0.5mg/kg per day for 12 weeks increased apparent collagen density in the lumbar spine and humerus, and had a greater trabecular bone surface to volume ratio (BS.BV) (203). The increased BMD may be due to decreased activity in the activation phase of remodelling or a positive bone balance in the BMU. Under optimum conditions the amount of bone removed by osteoclasts in the BMU is equally matched by new bone, allowing complete mineralisation. In osteoporotic bone this becomes imbalanced as resorption outstrips formation by the increased activation of new BMU's. This imbalance may be reversed to some degree with the use of alendronate (204). The drugs' anti-resorptive properties reduce the number of BMU's forming in the newly mineralised bone allowing for prolonged secondary mineralisation to be completed. Boivin *et al* (2000) collected biopsies from 53 postmenopausal osteoporotic women given alendronate for 3 years and found that the degree of secondary mineralisation improved compared to women on the placebo (205). They concluded that the level of increased mineralisation helped to explain the improved bone strength and reduced incidence of fracture in the patients on the alendronate therapy (205). In another study Roschger *et al* (2001) took transiliac bone samples from postmenopausal women who had been dosed with a continuous daily treatment of alendronate for 24 to 36 months. In this study they concluded that the size and shape of bone crystals were not affected by the use of alendronate. However the increased mineralisation throughout the bone samples was more uniform compared to the patients given the placebo (206).

Osteoporotic fractures in women commonly occur in areas such as the lumbar spine and hip. For some, taking alendronate at the onset of postmenopausal bone loss is seen as a protective method. Results have shown that the early use of alendronate prevented bone loss and maintained existing bone mass (207). However, in New Zealand, doctors prescribe alendronate to patients only after an individual has suffered osteoporotic fractures. In cases like these the introduction of alendronate caused a

reduction in the number and frequency of fractures in women who had already experienced vertebral fractures (208-210).

Fractures have been reported in patients after long-term use of alendronate in non-vertebral regions such as the femoral shaft (211-213). A theory offered to explain this, suggested that the reduced remodelling could delay or prevent micro-damage healing in the bone leading to the growth of cracks which would eventually cause fractures (211, 212). Abrahamsen *et al* (2009) questioned this in a recent evaluation of atypical fractures of the hip, subtrochanteric and proximal diaphyseal femur (214). They were unable to significantly differentiate between fractures suffered by patients taking alendronate long-term and fractures occurring in osteoporosis patients not taking the drug (214). After taking alendronate for 4 years women with low BMD but no recorded incidence of fractures had increased BMD and reduced fracture risk (215, 216), but the drug had no effect on women with high BMD (215). The use of alendronate slows or halts bone resorption in the elderly; however research has shown that stopping administration of the drug after long-term use can cause a gradual loss of effect on bone resorption (217, 218).

1.8.Goat milk and bone health

Milk is the major source of dietary calcium in the western world providing up to 70% of intake, and is generally seen as a positive factor in improving bone health (219). Traditionally cow's milk has supplied this market, and has been the focus of the majority of research on bone health. In the western world goat milk is viewed as a product that supplies a niche market. This market is mainly focused on providing a dietary calcium source to those individuals who have cow's milk protein allergies or lactose intolerance (220, 221). Consequently few studies have been conducted investigating the value of goat milk on bone health as either a promoter of bone growth, or to assist in alleviating the symptoms of bone aliments such as osteoporosis.

Milk is seen as a functional food, i.e. a food that provides a health benefit beyond that of its basic nutritional value. This is not exactly a definition in itself, as the term functional food has not been clearly defined in the scientific community. Functional foods do contain bioactive components which demonstrate a physiological benefit.

These benefits may include a reduction in the risk of some diseases such as osteoporosis.

The composition of goat milk and cow's milk are similar. They both contain the optimum ratios of calcium and phosphorus (2:1) needed for the enhanced absorption of these minerals into the body to maintain calcium homeostasis. However, goat milk does have lower amounts of vitamins such as foliate, vitamin B_6, and vitamin $B_{12,}$ but does contain higher levels of chloride and potassium. There are five major proteins in goat milk: α-lactalbumin, β-lactoglobulin, *k*-casein, β-casein, and α_{s2}-casein, which are also present in cow's milk (222). The main difference between the two types of milk is the amount of these proteins being present. α_{s1}- casein protein makes up approximately 55% of the proteins in cow's milk, whereas in goat milk the proportion is in some cases absent (222). This protein is one of several cow's milk proteins associated with common dairy intolerances and allergies (223). Disuse of dairy products due to allergies, lactose intolerance and/or misdiagnosis of these aliments, can lead to insufficient dietary calcium consumption (224). The resulting depressed bone mineralisation reduces 'Peak bone mass' and may increase the chances of developing osteoporosis either in children or the elderly (168, 224, 225).

1.8.1. Mechanisms affecting calcium absorption

Casein phosphopeptides

Casein contains the bioactive protein casein phosphopeptides (CPP) or mineral-binding phosphopeptides, which are formed from the digestion process in the stomach and small intestine (226-228). The phosphate residues appear as clusters and form into organophosphate salts, where it is been suggested they function as carriers for calcium across the distal small intestinal wall (229-231). This is done when CPP binds to calcium phosphate in a complex formulation (232). Bennett *et al* (2000) suggested that this process would be most effective with the CPP and calcium moving together from the stomach into the small intestine where passive calcium absorption takes place (227). CPP's increase calcium bioavailability by preventing calcium from interacting with other minerals (233).

Results from CPP enhancing calcium absorption and retention are mixed. Heaney *et al* (1994) reported better absorption in postmenopausal women with low absorption levels, when participants received the co-administration of calcium and CPP as part of a meal (234). In contrast, Lopez-Huertas *et al* (2006) found that the addition of CPP to calcium enriched milks did not significantly enhance calcium absorption in adult men and women (235). Animal studies have also given inconsistent results. One study indicated that ovariectomized rats given calcium bound CPP had decreased bone loss (236), but in other trials there was only a limited improvement, or no significant increases in absorption was found before absorption was reduced back to control levels (227, 237-239).

Goat milk fat

Due to its fat composition goats milk is considered to be more digestible for humans than cow's milk and has greater mineral absorption. Murry *et al* (1999) speculated "that the smaller sized fat globules in goat's milk facilitated greater digestion and absorption" (240). They also noted that due to the nutrient composition goats milk had a greater digestibility in pigs compared to cow's milk, and resulted in greater bone density as well as lower body fat (240). However, Jenness (1980) suggested that globule size was not the only factor; he referred to work done by Trout (1948) who found that reducing the size of cow's milk fat globules via homogenizing did not increase the digestibility of the milk compared to larger sized cow's milk globules (222, 241). Jenness (1980) went on to suggest that the higher percentage of short and medium chain fatty acids in goat milk fat compared to cow's milk fat increased the digestibility of goat milk in the small intestine (222). Triglycerides comprise 98-99% of goat milk fat and form into short, medium and long chain structures of which 95% are fatty acids (242). Milk fat is hydrolyzed by lipases in the small intestine and then solubilised by bile salts prior to being absorbed (243). Research has suggested that the lipase attacks the ester linkages of short and medium chain fatty acids more easily than long chain fatty acids, suggesting the length of the chain determines the digestibility of the fat globules (223, 244). In goat milk the percentage of more readily absorbed medium chain fatty acids in goats milk is 36%, compared to 21% in cow's milk (220, 245).

It should however be noted that not all milk from goats is alike; feeding regimes of the animals and different breeds do have an effect on the composition and the quality of the milk produced. Manipulations of goat feed has produced specialized foods, as is the case where diets were geared toward producing higher levels of unsaturated fatty acids; with the intention being to increase the amount of bioactive components in the milk (223).

1.8.2. Human studies

The human studies that investigated the value of goat milk as a nutritional supplement have only been reported in children. These studies evaluated the effectiveness of goat milk in comparison to cow's milk. None of these studies looked at bone health as the primary outcome; only as one of the parameters of overall body condition. One such study investigated the nutritional value of goat milk in girls and boys aged six to thirteen years in 1952. The children received either a glass of goat milk or cow's milk as part of their daily diet for five months. While both milks increased the overall body growth, the children who drank cow's milk had significantly increased growth measured using the 'Wetzel grid' method (i.e. a chart system that plots changes in the subjects height, weight, fitness, and related aspects of physical development). Those children who drank goats milk had significantly increased skeletal mineralisation (bone density using a Photoelectric Micophotometer), blood plasma vitamin A, and blood serum calcium compared to the children that drank cow's milk (246). Similar findings were shown in several other studies of malnourished children, where goat milk offered nutritional benefits on a par with that of cow's milk (247, 248).

1.8.3. Animal studies

The animal studies largely involved metabolic investigations of mineral bioavailability and absorption using the growing rat model. This work has been conducted mainly by a small group of investigators. They reported increased absorption of zinc, copper, selenium, calcium, iron and phosphorus in rats fed goat milk based diets compared to rats fed cow's milk based diets (249-251).

Several studies have directed their attention to investigating the nutritional value of goat milk in relation to several diseases. These studies have suggested that goat milk increases the bioavailability of iron in iron deficient rats (252, 253). However Park *et*

al (1986) concluded that while goat milk was superior to cow's milk, supplementation with ferrous sulphate was needed to show a positive increase in haemoglobin concentrations in anaemic male growing rats (253). In an investigation of malabsorption syndrome, rats underwent distal small intestinal (DSI) resection to restrict iron and copper absorption to induce anaemia. The removal of 50% of the intestine reduced the available absorptive area in the small intestine. This reduction caused an increase in the competition for limited binding sites, resulting in reduced absorption of both iron and copper (254). The development of anaemia was prevented due to higher digestibility and absorption of iron and copper in rats fed a goat milk diet compared to a cow's milk diet (249). Inducing malabsorption syndrome by resection in rats also allowed for the investigation into the digestibility of goat milk fat. Removing 50% of the DSI the researchers significantly limited the absorption of fat into the body. The results found that the rats fed the goat milk diet not only had greater digestive utilization of fat compared to the rats fed the cow's milk diet, but they also had lower cholesterol levels (245). They concluded that goat milk can be used as part of the diet for malabsorption syndrome (245).

Only one paper examined the effects of goat milk in relation to boss loss. The investigators found that the extra supplementation of the goat formulated diet with the increased level of calcium was able to significantly lift peak bone mass above the rats fed the control diet (255). The increased peak bone mass helped protect the rats from the ovariectomy induced bone loss, however they were unable to prevent this loss from occurring. Despite this, the ovariectomized rats fed the goat milk diet did maintain a significantly higher BMD and BMC in both the lumbar spine and femur compared to the ovariectomized rats fed the control diet (255).

1.9.Motivation and objectives of the thesis

Goat milk has been shown to increase the absorption of minerals in both animal and human trials, yet very little work has been done investigating the effects of goat milk in the field of osteoporosis, prevention of bone loss or bone architecture. It is important to seek prevention for future generations by increasing peak bone mass in children and young adults.

The co-administration of dietary supplements and medical treatments may also be complementary in their effects on osteoporosis. Therefore treatments that arrest bone loss in the femur of the ovariectomized rat may also allow greater mineralisation from dietary supplements such as goats' milk to take place. Milk is the major source of dietary calcium in the western world, and is generally seen as a positive factor in improving bone health. The bisphosphonate drug alendronate has been shown to be an effective tool in arresting bone resorption from the endosteal surface of osteoporotic bone in both animal and human trials. Considerable research has been done into the benefits of cow's milk in bone health, however very little attention has been given to the potential benefits of goat milk in this area.

The major objectives of the research presented in this thesis were:

1. To further the knowledge of how goat milk affects calcium bioavailability for optimum calcium absorption, growth and mineral accretion in the growing rat (Chapter 2 and 4).
2. To develop a method to determine regional morphological changes in the femur after ovariectomy (Chapter 3).
3. To investigate the effect of goat milk as a nutritional supplement with or without a medical treatment to determine any complementary effects on ovariectomy induced osteoporosis in the female rat (Chapter 5, 6, and 7), with regards to morphology, density and strength.

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2. The effects of formulated goats milk on calcium bioavailability in male growing rats.

There are two main proteins in milk; whey and casein. Casein contains casein phosphopeptides (CPP) which are released on digestion of the milk. These may increase calcium solubility by binding calcium in the small intestine. The increase of casein in the diet may help to stimulate bioavailability of calcium and increase bone density. Very little research has been done on goat milk in relation to bone health. This chapter attempted to further that knowledge by testing the hypothesis that altering the ratios whey and casein in the rat's diet will improve the bioavailability of calcium and increase bone density in growing male rats.

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2.1. Introduction

Milk is the major source of dietary calcium in the western world, and is generally seen as a positive factor in improving bone health. The composition of milk is believed to have optimal ratios of calcium and phosphorus (2:1) for the enhanced absorption of minerals, and the bioactive peptides found in milk protein are believed to play an active role in the bioavailability of calcium for absorption. Casein, one of the main proteins found in goat milk contains the bioactive protein casein phosphopeptides (CPP). It has been suggested that CPP increase calcium solubility (1, 2) by binding to calcium in the small intestine where dietary calcium absorption takes place (2, 3).

Goat milk fat may also contribute to increased calcium absorption. Previous work has indicated greater absorption of calcium with diets containing goat milk fat, compared to goat skim milk with added vegetable oils (4). It has been suggested that this is in-part due to a higher percentage of the more readily absorbed medium chain fatty acids found in whole goat milk (4-6). This study investigated the effect of different ratios of whey and casein and milk fat in goat milk formulations on calcium bioavailability in growing male rats.

2.2. Methods & materials

2.2.1. Animals

72 male Sprague Dawley rats, aged 3 weeks were randomly allocated into six treatment groups. The rats were housed in individual shoebox cages and weighed weekly. Rats from all treatment groups were kept at a constant temperature of 22°C (± 2°C) in a light-controlled 12/12 hour light/dark lighting regime.

2.2.2. Diets

All rats were maintained *ad libitum* on a semi-synthetic diet, with 50% of protein derived from egg albumin and the rest from goat milk. The goat milk protein consisted of three different ratios of 20:80 (Diet 1 and 4), 43:57 (Diet 2 and 5) or 83:17 (diet 3 and 6) whey:casein. Diets 1-3 had milk fat, whereas diets 4-6 contained a mixture of palm, coconut and soybean oil in proportions used in previous work calculated to provide a similar fatty acid profile to milk fat (4). All diets contained 10

g/kg corn oil to prevent essential fatty acid (EFA) deficiency. Vitamin and minerals known to affect bone growth and mineral accretion were balanced where possible including, calcium (0.5%), phosphorus (0.3%), and fat (~ 9%). All minimum dietary needs for growing rats were met as according to the AIN93G diet plan (7). Goat whole milk, goat skim milk, goat whey protein concentrate and goat fat powder where supplied by the Dairy Goat Co-operative, Hamilton, New Zealand. All rats were given *ad libitum* access to deionised water.

2.2.3. Metabolism experiment

The rats were housed in metabolism cages for 5 days at 7 weeks of age (week 4 of dietary treatment). Daily food intakes were measured, and urine and faeces collected for calcium and magnesium content. The quantity of calcium and magnesium absorbed and retained was determined by measuring total intake from diet and lost minerals via faeces and urine.

2.2.4. Duel energy X-ray Absorptiometry (DEXA) scans

In vivo duel energy x-ray absorptiometry (DEXA) scans were performed on anaesthetized rats at 8 and 12 weeks of age (week 5 and 9 of dietary treatment). DEXA was used to determine total body composition for comparisons between diets. Body composition is defined by whole body bone mineral density (g/cm^2) (BMD), bone mineral content (g) (BMC), fat mass (g), lean mass (g), mass of total body (g) and percentage of fat. Sub regional scans of mineral deposited in the L1-L4 lumbar spine and femur were measured using BMD and BMC.

Bone mineral measurements were taken using a Hologic Discovery A bone densitometer (Bedford, MA, USA). On each day that scans were undertaken, a quality control (QC) scan was taken to ensure that its precision met the required DEXA manufacturer's coefficient of variation. The coefficient of variation (CV) for the QC data was 0.98 – 1.01%. Each rat underwent three regional high-resolution scans of the spine and left and right femurs. Rats were positioned supine with right angles between the spine and femur, and between femur and tibia.

Coefficient of variance for the femurs ranged between 0.60% and 1.20% without and with repositioning between scans. These values ranged between 0.61% and 1.38% for the lumbar spine

2.2.5. Euthanasia and tissue collection

The rats were euthanized by exsanguinations under anaesthesia. Fasting blood samples were collected and the serum stored at -80°C. The lumbar spine and both femurs were removed by simple dissection and stored at -20 °C pending further analysis.

2.2.6. Bone marker

Blood serum was tested for analysis of the concentration of C-terminal telopeptides of type 1 collagen (CTx). This test was run using Ratlaps Elisa kits, supplied by Nordic Bioscience Diagnostic A/S, Herlev, Denmark.

2.2.7. Ashing and calcium content of femur

All adherent soft tissue was removed from left femurs and wet weights and lengths were recorded. Femurs were dried overnight at 105 degrees Celsius then ashed at 660 degrees Celsius overnight, both dry and ash weights were recorded. Ashed bones were dissolved in 2ml 6M hydrochloric acid (HCl) and then analyzed using Vistamodel Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES) machine (Varian, California, USA) for calcium analysis.

2.2.8. Biomechanical properties of the femur

Right femurs were thawed and any adherent soft tissue removed. The midpoint of the femur was marked and the width and thickness recorded using Mitutoyo vernier callipers (± 0.02mm) (Mitutoyo, Kawasaki, Japan). The bones were placed in phosphate buffered solution (PBS) and warmed to 23 degrees Celsius for 30 minutes in a water bath prior to mechanical testing. This temperature remained constant throughout the testing procedure. The biomechanical testing was done using the Shimadzu texture analyzer (Ezi test series) and results interpreted using Shimadzu WinAGSLite 2000 software (Shimadzu, Kyoto, Japan). The femurs were placed on the three point-bending jig with a fixed 12mm span, and subjected to a constant deformation rate of 50 mm min^{-1} with a 500N load cell.

2.2.9. Statistical analysis

Body weights, food intake, DEXA results, and bone markers were analysed using mixed models approach to repeated measures analysis (SAS 9.3, SAS Institute, Cary, USA) followed by Tukey HSD post-hoc test. The models included the effects of type of fat, whey/casein ratio, and time, as well as all their interactions. The change in BMC and BMD between 8 and 12 weeks of age was analysed for the effects of fat and whey/casein ratio using two-way analysis of covariance followed by Tukey HSD post-hoc test. The baseline result was included in the model as covariate.

. Biomechanics, femur size measurements, bone ash and calcium content results were analyzed using a one way ANOVA. Raw data was $\log_{10}$ transformed if necessary to achieve homogeneity of variance. . The metabolism data were pooled over the 3 days of the experiment. All results were expressed as the means and standard deviation (SD), and statistical significance was set at $p<0.05$.

2.3. Results

Table 2-1 shows the analysis for the various diets. Minerals, fats and proteins for the six formulated diets in the trial were balanced. No significant differences were found between diet intake and rat bodyweights between treatment groups throughout the trial (data not shown).

Table 2-1. Analysis of diet composition of the six formulated goat milk diets.

Diet composition	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
N %	3.3	3.2	3.2	3.2	3.3	3.4
Crude protein %	20.3	20.3	20.1	19. 8	20. 7	21.0
Protein from casein % [1]	39	28	8	40	28	8
Dry matter %	94.0	94.1	94.3	93.9	92.5	94.4
Ash %	5.4	5.4	5.5	5.2	5.4	4.6
Fat %	10.0	10.1	9.5	8.8	8.6	9.1
Fat from milk fat % [2]	86	85	92	4	7	11
Carbohydrates %	58.2	58.3	59.3	60.1	57.8	57.3
Calcium (g kg^{-1})	5.1	5.3	5.8	5.4	5.5	6.3
Magnesium (g kg^{-1})	1.5	1.5	1.4	1.5	1.5	1.4
Phosphorus (g kg^{-1})	3.4	3.3	3.3	3.5	3.4	3.5

Diet 1, protein ratio 20:80 whey: casein with milk fat; Diet 2, protein ratio 43:57 whey: casein with milk fat; Diet 3, protein ratio 83:17 whey: casein with milk fat; Diet 4, protein ratio 20:80 whey: casein with vegetable oils; Diet 5, protein ratio 43:57 whey: casein with vegetable oils; Diet 6, protein ratio 83:17 whey: casein with vegetable oils.
[1] Calculated from the amount of goat casein divided by the total protein from the dietary analysis. [2]Calculated from the amount of goat milk fat divided by the total fat content from the dietary analysis.

2.3.1. Mineral balance

The rats fed the diets containing the lowest casein had significantly lower absolute calcium absorption ($p<0.0005$) compared to those rats fed the diets with 57% and 80% of the goat milk protein as casein. This was also reflected in fractional calcium absorption (Fig. 2-1). Intake of calcium did not differ between diets; however excretion levels in the rats fed the lowest casein diets were significantly greater. The rats fed diet 3 had significantly higher calcium excretion from the urine ($p<0.0005$) compared to all other rat groups, whereas the rats fed diet 6 with lowest casein and no goat milk fat had significantly higher calcium excretion from the faeces ($p<0.0005$) against all other groups. Magnesium intake differed significantly between the rats of the different diet groups and no obvious pattern of effect was found. The rats fed diet

3 had significantly lower magnesium excretion from the faeces ($p<0.0005$) against all other rat groups except diet 6 (data not shown).

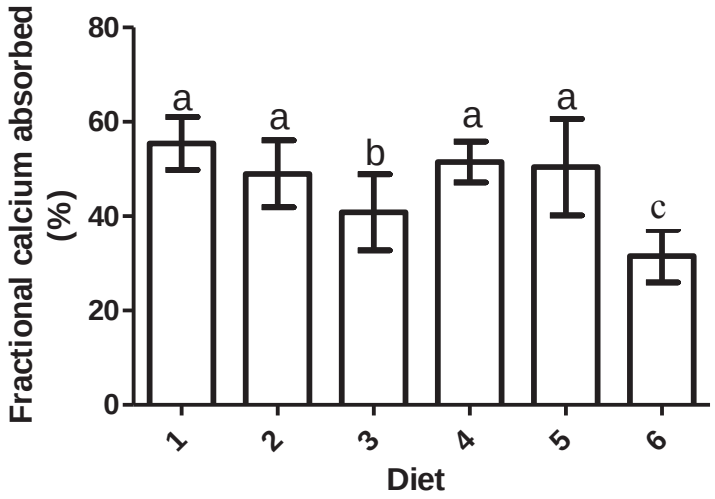

Figure 2-1. Fractional calcium absorption (%) and SD from the metabolism trial at seven weeks of age, for the six diets, (n = 12 for each diet).

Diet 1, protein ratio 20:80 whey: casein with milk fat; Diet 2, protein ratio 43:57 whey: casein with milk fat; Diet 3, protein ratio 83:17 whey: casein with milk fat; Diet 4, protein ratio 20:80 whey: casein with vegetable oils; Diet 5, protein ratio 43:57 whey: casein with vegetable oils; Diet 6, protein ratio 83:17 whey: casein with vegetable oils. Values with different letters are significantly different ($p< 0.05$) between diets.

2.3.2. DEXA

No significant differences were found between whey: casein ratios or type of fat used; nor was there any correlation between the ratios of whey: casein and the type of fat used in the diets at either 8 or 12 weeks of age. Investigation of changes between the two time points (8 and 12 weeks of age) showed a significant difference in BMC ($p< 0.03$) and BMD ($p< 0.04$) in the lumbar spine region (Fig. 2-2). Post hoc testing showed rats fed diet 5 had a significantly higher BMC ($p<0.03$) than rats fed diet 4 in the lumbar spine region. Post hoc testing showed that the rats fed diet 4 had a trend towards having lower lumbar spine BMD ($p<0.08$) than the rats fed diets 3 and 5. No significant differences were noted in the other parameters and body sites tested with bone densitometry (data not shown).

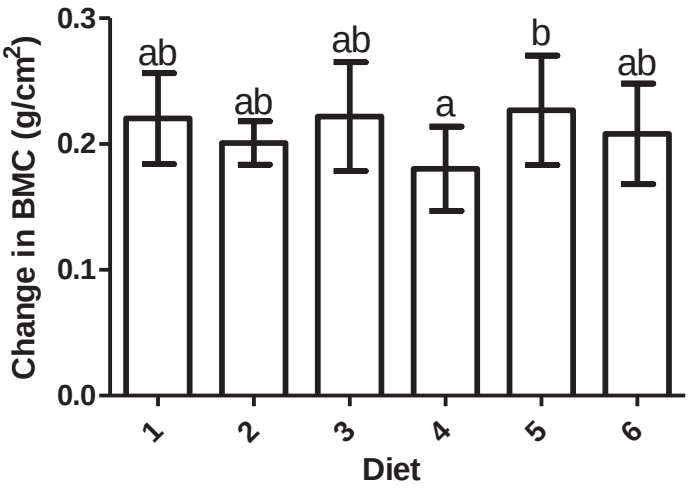

Figure 2-2. Means and SD for the change in Lumbar spine BMC between the two time points (8 and 12 weeks of age) for the six diets (n = 12 for each diet).

Diet 1, protein ratio 20:80 whey: casein with milk fat; Diet 2, protein ratio 43:57 whey: casein with milk fat; Diet 3, protein ratio 83:17 whey: casein with milk fat; Diet 4, protein ratio 20:80 whey: casein with vegetable oils; Diet 5, protein ratio 43:57 whey: casein with vegetable oils; Diet 6, protein ratio 83:17 whey: casein with vegetable oils. Values with different letters are significantly different ($p < 0.05$) between diets.

2.3.3. Ex vivo femur

Examination of the femur ex vivo showed that the variations in whey and casein ratios had no significant effect on the size of the bone or on biomechanical strength, ash content, and calcium content (Table 2-2).

2.3.4. CTx

Investigations of the bone marker for resorption revealed no significant differences between serum concentration levels (ng/mL) of CTx in the rats fed the six formulated diet (Table 2-2).

Table 2-2. Size measurements, dry weight, ash content, calcium content and biomechanical testing for femurs, as well as concentration levels (ng/mL) of serum C-terminal cross-linking telopeptide of type I collagen (CTx), for the six diets.

	Diet 1 N = (12)	Diet 2 N = (12)	Diet 3 N = (12)	Diet 4 N = (11)	Diet 5 N = (12)	Diet 6 N = (11)	p value
Femur length (mm)	35.5± 0.60	35.5 ± 0.86	35.3± 0.89	35.6 ± 0.75	35.3± 0.84	35.6± 0.93	0.862
Femur thickness (mm)	3.5 ± 0.19	3.6 ± 0.20	3.5± 0.18	3.5± 0.19	3.6± 0.18	3.5 ± 0.30	0.558
Femur width (mm)	4.7 ± 0.34	4.8± 0.24	4.8± 0.25	4.7 ± 0.32	4.8± 0.29	4.7 ± 0.31	0.876
Ash (%)	51± 4.0	52± 3.1	53± 3.6	51± 4.2	50± 3.6	49± 2.3	0.369
Calcium (mg/g)	189± 16.1	192± 21.6	197± 13.7	193± 18.9	189 ± 13.5	188± 8.2	0.451
CTx (ng/mL)	15± 2.1	17± 3.4	17± 2.3	16± 2.9	16± 2.4	15 ± 2.2	0.380
Max Load (N)	183 ± 15.1	181± 15.7	176 ± 16.7	173± 11.6	176± 18.7	177± 20.7	0.720
Break load (N)	182 ± 15.5	170 ± 23.8	172± 17.2	172± 12.1	168± 34.2	176± 19.5	0.679
Elasticity (N/mm^2)	545± 138	472 ± 92	526 ± 117	528 ± 137	484± 107	540 ± 174	0.654
Energy (J)	0.176 ± 0.02	0.187 ± 0.03	0.17 ± 0.02	0.174 ± 0.02	0.177 ± 0.03	0.166 ± 0.03	0.537

Values are mean ± SD, with statistical significance was set at ($p< 0.05$). Diet 1, protein ratio 20:80 whey: casein with milk fat; Diet 2, protein ratio 43:57 whey: casein with milk fat; Diet 3, protein ratio 83:17 whey: casein with milk fat; Diet 4, protein ratio 20:80 whey: casein with vegetable oils; Diet 5, protein ratio 43:57 whey: casein with vegetable oils; Diet 6, protein ratio 83:17 whey: casein with vegetable oils.

2.4. Discussion

This study investigated various ratios of whey and casein in goat milk formulations and their subsequent effect on calcium bioavailability in growing male rats. Over the nine week period of the trial the diets containing 80% and 57% of goat milk protein as casein increased calcium and magnesium absorption compared to the diets containing only 17% casein. These results suggest a minimum level of casein is needed to optimise calcium absorption from goat milk. The difference found between levels of casein and apparent calcium absorption seen in this trial could be explained by the theory postulated by Erba *et al* (2002) (8). In their study, Erba *et al* (2002) investigated different ratios of casein phosphopeptides and calcium on passive calcium transport in the small intestine, and suggested that there was an optimum CPP/calcium ratio of 15 for increased absorption (8). However, in the current trial increased calcium absorption did not result in significant differences in bone mineral content or density. Nor was it reflected in *ex vivo* ash (mineral) content and calcium content of the femurs, suggesting that the varying ratios of protein tested did not affect mineral uptake or retention into the femur during the period of this trial. This was also the case with the biomechanical results indicating that there was no effect on bone strength.

This appears to be in line with some reported findings, where studies investigating casein levels in cow's milk noted that changes found in increased calcium absorption were temporary. Bennett *et al* (2000) found that a high casein diet enhanced calcium absorption, although the efficiency of the calcium absorption had reduced within 2 weeks, whereas other studies found no effect at all (9). Howe and Beecher (1981) found that on a diet of 9% calcium and 3.5% phosphorus, an increase in protein as casein from 25% to 45% did not have an effect on calcium absorption in young growing rats (10). Yuan and Kitts (1994) found a decrease in feed efficiency, femur calcification and physical measurements from the group fed a low casein diet (60g/kg) compared to the control group on 200g/kg of casein (11). They however did not find any difference between levels of casein and calcium absorption. Allen and Hall (1978) investigated the effects of two different levels of protein 18% and 36% on

calcium absorption, with casein as the protein source (12). They found that there was no difference in absorption, bone turnover or bone calcium.
One study found was referring to the effects of CPP on calcium absorption from goat milk in rats. This study compared the effects of adding CPP from bovine and caprine sources to calcium fortified milk. They concluded that both sources of CPP increased calcium absorption when added to calcium fortified milks (13).

The vegetable oils used as a fat source in the diets may have also reduced mineral absorption compared to the diets with whole goat milk. Diet 6 containing the highest levels of vegetable oils had the lowest levels of calcium absorption in the study. Kruger *et al* (2008) using the same mix of vegetable oils found that the diet containing whole goat milk had significantly greater absorption of calcium than the diet containing goat skim milk powder and vegetable oils (4). They theorized, as did Lopez Aliaga *et al* (2000) that this is in part due to a higher percentage of the more readily absorbed medium chain fatty acids found in goat milk (14). This theory was also shared by Nestares *et al* (2008) on the bioavailability of magnesium, where they found that anaemic rats fed goat milk had greater mineral absorption (15). Oddly, in terms of bone mineral retention the only significant difference found was between two diets using vegetable oil as their fat source. Although neither diet was significantly different to any of the other diets, it would suggest that during the later stages of growing male rats a casein level of 57% was more advantageous than 80% in the lumbar spine. However, any differences in BMC were lost by peak bone growth.

This still leaves the question concerning the lack of calcium retention in bone in the rats with greater calcium absorption. It could be suggested that the mechanisms that allow for greater calcium absorption became less efficient over time allowing for the less bioavailable diets to catch up, thereby introducing balanced results between the diets tested (9).

2.4.1. Conclusion

This study found that the diets containing 80% and 57% of goat milk protein as casein delivered increased calcium absorption compared to the diet containing the lowest level of casein, suggesting a minimum level of casein is needed to optimise calcium

absorption from goat milk. However increased calcium absorption did not appear to impact mineral uptake or retention in the femur within growing male rats. The mechanism to explain this lack of retention remains unclear.

These results add to the current knowledge in the area of manipulating goat's milk formulas to improve the bioavailability of calcium for absorption and retention. The results of this study also show that altering the casein levels of goat's milk has similar outcomes to cow's milk formulations that have comparable manipulations.

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3. The effects of ovariectomy on the architecture of different regions within the femoral shaft of female rats.

Direct sectioning is a method of investigating area changes in the architecture of cortical bone. This chapter used this method to examine regional changes in the morphology of the rat femoral shaft as a result of ovariectomy surgery. This chapter forms the basis for further bone regional analysis of the follow up studies.

3.1. Introduction

Osteoporosis causes changes in femoral shaft geometry resulting from uneven bone turnover. The size of the medullary cavity is exponentially increased due to the reduced rate of bone formation to bone resorption at the endosteal surface. This results in thinning of the cortical wall in the femoral shaft. The subsequent loss of bone impairs the strength of the whole structure, as well as reducing the strength of the bone material (1). Bone responds to this weakening of the structure by laying down new bone matrix on the outer surface on the bone. The increased stresses placed on the thinning shaft stimulate bone cell activity resulting in bone formation at the periosteal surface, therefore, increasing the circumference (size) of the femur (2, 3). This increase in bone circumference strengthens the whole femur, which in turn helps to preserve the bone's structural strength. However, while this periosteal growth has been shown to improve the bending strength of the femur, it does not completely compensate for what was lost (4); nor is the degree of osteoporotic periosteal apposition equivalent to that of growing bone (5).

Historically the femur diaphysis, consisting largely of cortical bone, has received less attention than the femoral neck and distal regions of the femur. This is probably due to the more noticeable effects of osteoporosis on trabecular bone, and the higher instance and more typical fractures occurring in the femoral neck region (6, 7). However, the effects of osteoporosis on the mechanical competence of cortical bone should not be overlooked. Cortical bone provides structural rigidity and plays a significant role in the mechanical ability to cope with load (8).

The ovariectomized rat model is often used in pre-clinical trials to create osteoporotic like conditions to test potential treatments (9). The bone mineral density, bone mineral content, and gross bone area effects of these treatments are often assessed by Dual energy x-ray absorptiometry (DEXA). However, this method alone does not offer insight into the effect of treatments on geometric changes within the structure of the bone. The contribution of changes to the bone structure can be assessed using Peripheral quantitative computed tomography (pQCT) and Micro-computed tomography (μCT) (10-12). These methods can, unfortunately, be difficult to access and are expensive (13). More traditional methods of acquiring area measurements are

available and have been shown to provide accurate data such as radiograph imaging, latex casting, or direct sectioning (7, 13-15).

The purpose of this preliminary study was to determine the effects of ovariectomy on the form, mineral density and longitudinal compression strength of the femoral shaft (in the 28 week old Sprague Dawley female rat). Also, to determine if the direct sectioning method used could detect the geometric changes known to accompany ovariectomy in the mature female rat. This was achieved by separately assessing these parameters in a series of consecutive slices of the femoral shaft that extend distally from the tip of the third trochanter to the proximal portion of the intercondylar ridge.

3.2. Methods & materials

3.2.1. Animals

42 Sprague-Dawley female rats were randomly allocated into 2 groups, ovariectomized (OVX) and sham (SHAM) operated rats. The rats were anaesthetised for surgery by inhalation of 1.5 - 2% isofluorane (Nicholas Piramal Ltd, Merial). A midline incision was made half way between the hump of the back and the base of the tail. The skin was pulled half way down one side of the body and an incision made through the peritoneal muscles. The ovaries were exteriorized and the fallopian tube, uterine horn, ovarian blood vessels and fat were severed with a single cut through the distal part of the horn, and the horn returned to the abdominal cavity. A similar procedure was used for sham surgery, except that the ovaries were not exteriorised or removed. Surgeries were performed at 28 weeks of age. . Rats from the two treatment groups were individually caged and maintained in the same temperature-controlled room at a constant 22° C in a 12/12 light/dark lighting regime, and weighed weekly.

3.2.2. Diets

All rats were maintained on the same pellet diet for the duration of the experiment. The protein content of the diet was 19%, calcium 1.1% and phosphorus 0.78%. This diet fulfilled National Research Council minimum dietary recommendations for

laboratory animals (16). Dietary intake was restricted to 17g/day in order to prevent the rats from becoming obese (17). All rats were given *ad libitum* access to deionised water throughout the study.

3.2.3. Bone densitometry by Duel energy X-ray Absorptiometry (DEXA)

The regional BMD, BMC, and area of the excised femurs were determined by DEXA using Hologic QDR4000 scanner with a regional high-resolution pencil beam (Hologic). Quality control (QC) scans were done at the start of each day of scanning; the coefficient of variation (CV) for QC's was 0.98 – 1.0%, the CV for the femurs was between 0.92 – 0.85%.

Each rat underwent three regional high-resolution scans of the spine and left and right femurs. Before scanning the rats were anaesthetised with a drug mixture of Ketamine, Acepromazine (ACP), sterile water, and Xylazine at a ratio of (5:2:2:1). The dose was administered via intra-peritoneal injection at 0.05ml/100g of body weight. Rats were positioned supine with right angles between the spine and femur, and between femur and tibia.

3.2.4. Euthanasia and tissue collection

19 weeks after ovariectomy surgery the rats were euthanized by CO_2 inhalation. Both hind legs of each rat were subsequently removed by simple dissection, and placed in phosphate buffered saline (PBS) at -20° C pending further analysis. The uteri were also removed and weighed in order to verify that ovariectomy had been successful. The femurs were subsequently removed from the hind legs by simple dissection, their weight and length being determined after any residual adherent soft tissue had been removed.

3.2.5. Right femurs

Each right femur was fixed in 10% formalin for 24 hours and subsequently dehydrated by passing through increasing concentrations of ethanol. Each femur was then soaked in Xylene, at room temperature for 24 hours, to remove any fat before being positioned in labelled rectangular 45mm x 10mm cuvettes to be embedded in Epoxy resin (Ados epoxy resin, CRC Industries, NZ). Prior to the filling of each

cuvette with resin, the contained femur was positioned with its long axis lying exactly parallel to that of the cuvette. A line corresponding to the midpoint of the intercondylar notch was then drawn lengthwise, in permanent ink, along the outside of the cuvette so as to provide a radial reference point for each slice of bone after subsequent cutting. Each cuvette was stored for a week to allow the resin to completely set prior to sectioning. The surface of each cuvette was then marked with a series of transverse lines that were spaced so as to divide the length of each femur into 20 transverse slices, each comprising 5 % of the overall length. Each resin block and the containing plastic cuvette was then sectioned along the transverse lines with a diamond wheel saw. The distal surface of each slice was marked immediately after cutting so as to provide longitudinal orientation, before being placed in a labelled container pending further analysis. Subsequent measurements were taken from the eleven slices (slices 5-14) that constituted the tubular portion of the femoral shaft. These slices spanned 55% of the overall length, and extended from the upper limit of the intercondylar ridge (slice 5) at the distal end of the femoral shaft to the upper limit of the third trochanter (slice 15) at the proximal end of the femoral shaft.

Each femoral slice was scanned with Epson Perfection 3200 Photo colour scanner to a resolution of 2400 dpi. The inner and outer edges of the bone profile of each slice were enhanced by image analysis software (Ulead Photoimpact 6 software, Ulead Systems Inc, Taipei, Taiwan). The bone area, total cross sectional area and marrow cavity area (Fig. 3-1) of the enhanced upper and lower images of each slice image was determined using an engineering software package (Image J, U. S. National Institutes of Health, Maryland, USA). The cross section moment of inertia (CSMI) of each slice was determined using the engineering software package, Area properties (Freeware, softpedia.com).

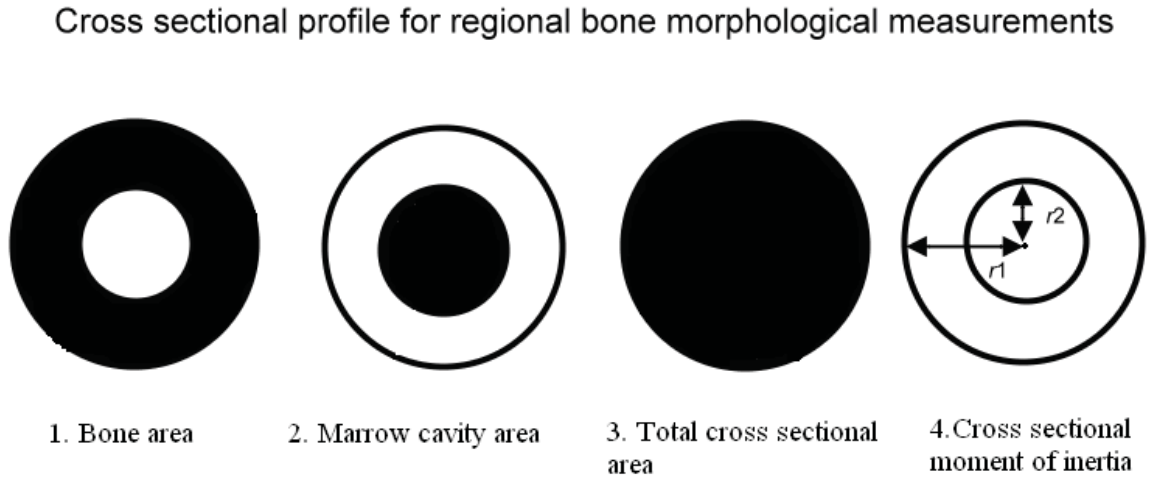
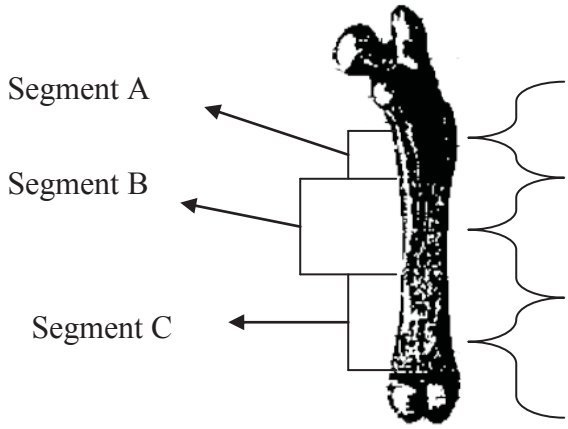


Figure 3-1. Picture describing the cross sectional profile of the femur slice.
1. Shows the area used to calculate the bone area (mm^2).
2. Shows the area used to calculate marrow cavity area (mm^2).
3. Shows the area used to calculate the total cross sectional area (mm^2).
4. CSMI of a circular mass is calculated using CSMI = $(\pi/4) \times (r_1^4 - r_2^4)$.

3.2.6. Ash content of femoral slices

A pilot study had shown that the mass of ash obtained from individual slices was so low as to accrue significant errors in weighing. Therefore consecutive femoral slices were grouped into 3 femoral 'segments' (Fig. 3-2) and each group ashed at 660^{o}C. Segments A, B and C corresponded to the regions of bone lying between: the upper border of slice 15, the lower border of slice 13, the upper border of slice 9 and the lower border of slice 5 respectively. The mineral composition (relative to the amount of organic and inorganic material: g/g air dry bone mass) of each segment was calculated by dividing the ash content (grams) by air dry weight (grams) of the segments.



Slice	Mean bone area (mm^2)
15	9.64
14	9.02
13	8.22
12	7.78
11	7.59
10	7.32
9	7.08
8	6.86
7	6.70
6	6.46
5	6.56

Figure 3-2. Diagram of rat femur showing the segments A, B and C in relation to slice numbers.
The slices of the femoral shaft each comprised 5% of the total length of the femur. The slices were grouped into sections by similar mean bone area, as follows: 13-15 (group A), 9-12 (group B) and 5-8 (group C).

3.2.7. Left Femurs

Each left femur was transversely sectioned at points corresponding to the three lengthwise 'segments' of the shaft used for determining mineral composition in the right femur (Fig. 3-2). Each shaft was sectioned at positions corresponding to: the upper border of slice 15, the lower border of slice 13, the upper border of slice 9 and the lower border of slice 5 respectively. The upper and lower profiles of each of the three sections were each scanned and their cross sectional areas determined in the manner described above. All sections were kept moist with phosphate buffered solution (PBS) during these procedures and subsequently stored in PBS pending biomechanical testing.

3.2.8. Biomechanical properties of the Left femoral segments

The ends of each of the three femoral segments from the left femurs were polished with extra fine sandpaper so that their surfaces would lie flush with the compression plates of the texture analyser. All segments were examined prior to testing and any cracked segments were discarded. The maximum load (N) and extrinsic stiffness (N/mm) (structural strength) of each segment were then determined on compression in a 10kN load cell on an Instron 4502 texture analyser. The stiffness was determined

from the slope of the linear portion of the force deformation curve, and was based on a best fit linear regression.

3.2.9. Statistical Analysis

Data were analysed in the statistical package "SYSTAT" (version 11) (Systat, Chicago, USA) and displayed in tables and text as means ± standard deviations (SD). The mean bone area, mean marrow cavity area and mean total cross sectional area of each of the 11 slices taken from the shaft of each right femur were each normally distributed and, therefore, were amenable to parametric analysis. Body weight, uterus weight, DEXA femur area, BMC and BMD were also normally distributed and were analyzed by one way ANOVA. Mineral composition was found to have non-normal data and log transformation was unsuccessful in normalizing the data's distribution. Therefore the mineral composition data were normalized using a Johnson transformation (18), with the statistical package "MINITAB" (Minitab Inc, Pennsylvania, USA). The data for mineral composition were transformed using

$$Y=3.10394 + 2.43925 * \text{Asinh}[(X-0.685175)/ 0.0365547]$$

The mineral composition, maximum load and extrinsic stiffness of the three segments were then analyzed using a two-way ANOVA.

The effects of ovariectomy and sham ovariectomy on overall bone area (mm^2), marrow cavity area (mm^2) and total cross sectional area (mm^2) in the femoral shaft were each compared by one way repeated measure ANOVA. The pattern of variation of each of these parameters between the slices that make up the femoral shaft was explored using multivariate principal component analysis (PCA). A one way ANOVA was conducted on the individual PC scores in each axis of variation (PC1 and PC2) in order to statistically compare the effects of the two treatments.

The data from bone samples damaged during preparation (i.e. broken or cracked during cutting) were removed from the analysis.

Principal component analysis

PCA is used to identify the structure of the data i.e. the principal components in the data. It is a technique that may reduce the dimensions of the data from a larger group

of correlated variables to a smaller set of uncorrelated variables. For example, a researcher is looking at changes in the thickness of tree trunks, and does this by measuring 60 variables along the length of a tree trunk in a grove of trees. Instead of looking at 60 different variables the PCA looks for a smaller set of components, say 'size' and 'shape' to explain the variation. The smaller set of components are organised into order of decreasing relevance .i.e. the first principal component (PC1) accounts for most of the variation in the original data, then the second principal component (PC2) accounts for the next largest group of variation not accounted for in the first principal component, and so on, until 100% of the original set of 60 variables are explained. I.e. PC1 = 70%, PC2 = 20%, PC3 = 8%, and PC4 = 2% totalling 100%. Realistically most researchers opt to stop the analysis before the total variance is explained concentrating their investigation on the first few components that explain the majority of the total variance. Determining when to stop the analysis is typically done following several rules; the first is to consider using only those PC's whose cumulative value explains 70% to 80 % of the total variance. The second is Kaiser's stopping rule – where only PC's (also known as eigenvectors) with an eigenvalue of greater than 1 are retained (19), meaning, that you should not consider using an eigenvalue that has less variance than one of the original variables. The third is to examine a Scree plot and identify where the 'elbow' in the data is located (Fig. 3-3). I.e. all data points after the 'elbow' explains only small amounts of the variance, and all data points above the 'elbow' explain the largest amount of the variance. Lastly, the forth rule to consider is whether the selected PC's provide a useful explanation of the variance.

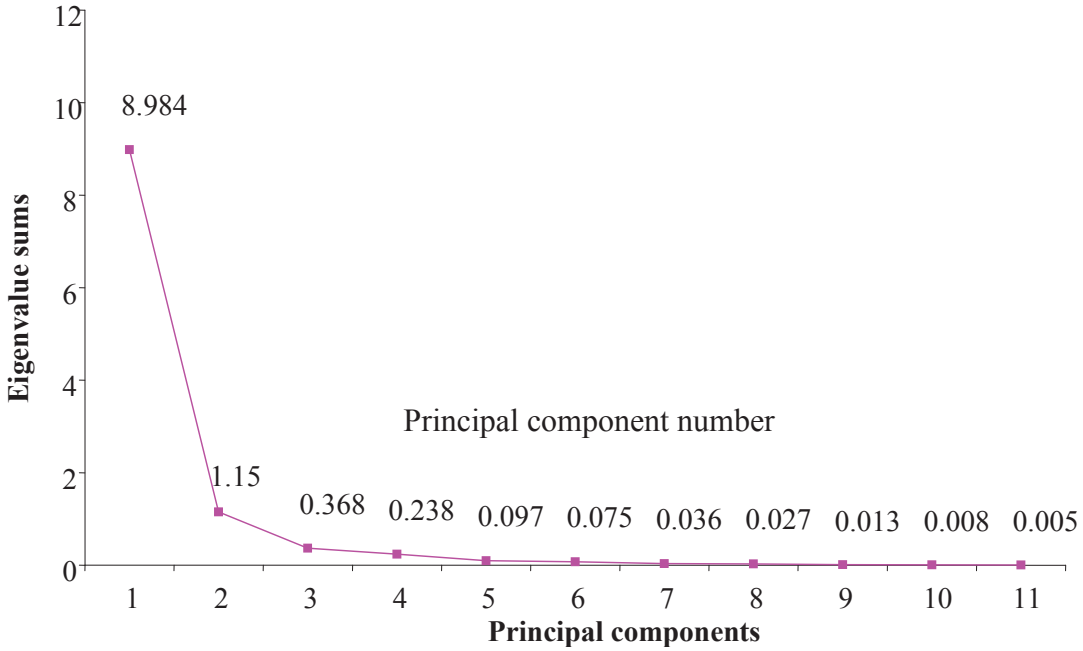


Figure 3-3. Scree plot of the cumulative eigenvalue sum versus the number of principal components.

The selected PC's are displayed as component loadings, and show how each of the variables are related to the components (20). The first may show that each of the variables is large and positively correlated to the component. Therefore, the first component could be considered a measure of overall size or 'thickness of the tree trunk'. I.e. trees with large dimensions would score highly on this component and trees with small dimensions would score low on this component. The second component may have variables that are large but are either positive or negative. This suggests a contrast between these positive and negative variables and, therefore, it must be considered what commonalities exist between these subsets. In the case of the tree dimension there might be contrast between different regions of the tree indicating a change in shape, i.e. the trunk of taller thin trees may not vary in shape, whereas short trees may be thicker at the base and thinner at the top. Components may also include variables with very low numbers (close to zero); these indicate variables with a low correlation to the component.

3.3. Results

Over the course of the trial we had two rats die under anaesthesia due to what could have been an adverse reaction to the anaesthetic drugs used during DEXA scanning. The uterus weights of OVX rats (0.117g ± 0.008) were all below SHAM rats (0.802g ± 0.028) confirming ($p<0.05$) the ovariectomy surgeries had been successful. The mean body weight of OVX rats at euthanasia (434.82g ± 22.99) was significantly greater ($p <0.05$) than SHAM rats (384.96g ± 26.41). The OVX rats had a tendency to have longer femurs (35.3mm ± 0.88) compared to SHAM rats (34.8mm ± 0.68) on ANOVA ($p<0.071$).

3.3.1. Morphology of the femoral shaft

Bone area (mm2)

There were no significant overall differences between repeated measures ANOVA of OVX and SHAM rats in the bone areas of the eleven slices of the shaft of the femur (Table 3-1b). There were, however, significant differences between slices from different locations ($p< 0.0005$). Further, the pattern of variation between slice locations differed significantly between OVX and SHAM rats ($p<0.0005$). Therefore, while ovariectomy did not influence the overall bone are of the femoral shaft it significantly influenced the pattern of distribution of bone area along the femoral shaft.

Principal component analysis (PCA) was then used to determine where in the shaft those differences in bone area occurred, and where there was a treatment effect. Table 3-2 gives the results of the PCA of the 15 slices that made up the femoral shaft. A total of two principal components were sufficient to explain 91% of the variation in the original data.

The effect on treatment on each of the PC scores was investigated by one-way ANOVA. PC1 scores showed there was no significant difference between treatments, whereas, PC2 scores of OVX rats were significantly higher than SHAM rats ($p< 0.0005$). Therefore, PC2 showed that there was a significant contrast in bone areas between treatments in different regions of the shaft. The bone areas in the proximal region of the femoral shaft of OVX rats were relatively smaller than SHAM rats, and

the bone area of OVX rats in the distal region of the shaft were larger than SHAM rats.

Table 3-1. Means, SD's and repeated measures ANOVAs of overall bone area (mm^2), marrow cavity area (mm^2), and total cross sectional area (mm^2) of slices of the femoral shaft from ovariectomized and sham operated rats.

a)

Parameter	OVX		SHAM	
	N	Mean ± SD	N	Mean ± SD
Bone area (mm^2)	16	84.7 ± 6.81	17	81.9 ± 7.01
Marrow cavity area (mm^2)	16	56.8 ± 7.21	17	47.3 ± 3.62
Total cross sectional area (mm^2)	16	142 ± 11.7	17	129 ± 8.4
Cross sectional moment of inertia (mm^2)	16	273 ± 42.7	16	236 ± 33.9

b)

Bone area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	1,31	1.325	0.259
Within subjects			
Slice	10,310	292.038	**0.0005**
Slice*Treatment	10,310	7.111	**0.0005**
Marrow cavity area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	1,31	23.219	**0.0005**
Within subjects			
Slice	10,310	570.260	**0.0005**
Slice*Treatment	10,310	5.580	**0.003**
Total cross sectional area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	1,31	12.032	**0.002**
Within subjects			
Slice	10,310	259.596	**0.0005**
Slice*Treatment	10,310	0.522	0.875
Cross sectional moment of inertia (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	1,31	7.585	**0.010**
Within subjects			
Slice	10,300	185.344	**0.0005**
Slice*Treatment	10,300	1.491	0.142

Table 3-2. Means and SD's of the bone areas of consecutive slices from the femoral shafts of ovariectomized and sham operated rats and results of principal component analysis.

	Bone area (mm^2)					
	OVX		SHAM		Component loadings	
Slice*	N	Mean ± SD	N	Mean ± SD	PC1	PC2
15	18	9.5 ± 0.86	17	9.8 ± 1.04	0.87	-0.46
14	19	9.0 ± 0.89	17	9.1 ± 0.97	0.86	-.039
13	18	8.2 ± 0.78	17	8.3 ± 1.01	0.88	-0.40
12	19	7.7 ± 0.80	17	7.8 ± 0.69	0.90	-0.29
11	19	7.7 ± 0.63	17	7.5 ± 0.85	0.94	-0.20
10	18	7.5 ± 0.62	17	7.2 ± 0.57	0.97	0.03
9	19	7.2 ± 0.70	17	6.9 ± 0.53	0.93	0.17
8	19	7.0 ± 0.52	17	6.7 ± 0.57	0.95	0.25
7	19	6.8 ± 0.55	17	6.5 ± 0.55	0.93	0.314
6	19	6.7 ± 0.62	17	6.1 ± 0.47	0.83	0.48
5	19	6.9 ± 0.52	17	6.2 ± 0.41	0.72	0.57
Percent of Total Variance Explained					**78.65**	**12.59**

**Slice 5 is located distally on the femoral shaft just above the condyle ridge; slice 15 is located proximally at the lower limit of the third trochanter. The thickness of each slice is 5% of the overall bone length.* Principal component analysis (PCA) showed two axes of variation in bone areas. The first principal component, axis PC1 accounted for 78.6% of the variance, all component weightings having large positive values (Table 2.3), indicating variation in overall size. The second principal component, PC2 accounted for 12.5% of the variance and reflected a contrast between bone areas in the proximal and distal ends of the shaft i.e. a difference in the pattern of distribution of bone area along the femoral shaft.

Marrow cavity area (mm^2)

The overall marrow cavity areas of the slices from the femoral shaft were significantly greater on repeated measures ANOVA ($p < 0.0005$) in the OVX rats than SHAM rats (Table 3-1). There was significant variation on ANOVA between slices in marrow cavity area ($p < 0.0005$) and the presence of a significant interaction term ($p<0.003$) indicated there were significant differences between treatments in the pattern of variation of the marrow cavity area. Therefore the overall marrow cavity area of the femoral shaft was relatively increased in OVX rats, and the pattern of increase within slices along the length of the femoral shaft differed from that of SHAM rats.

Table 3-3 gives the results of the PCA of the 15 slices that made up the femoral shaft. A total of two principal components were sufficient to explain 89% of the variation in the original data. The effect on treatment on each of the PC scores was investigated

by one-way ANOVA. The PC1 scores of OVX rats were significantly greater than those of SHAM rats ($p<0.0005$), i.e. a greater overall cavity area than SHAM rats. Further, a one way ANOVA of the PC2 scores showed a difference of borderline significance ($p< 0.061$) between the two treatment groups. This suggested, in conjunction with the results from the repeated measures ANOVA of the parameters, that there was a tendency for the marrow cavity area of proximal femoral shaft slices of OVX rats to increase and the marrow cavity area of the distal slices to decrease relative to SHAM rats.

Table 3-3. Means and SD's of the area of the marrow cavities of consecutive slices from the femoral shafts of ovariectomized and sham operated rats and results of principal component analysis.

	Marrow cavity area (mm^2)					
	Ovariectomized rats		Sham operated rats		Component loadings	
Slice*	N	Mean ± SD	N	Mean ± SD	PC1	PC2
15	18	4.7 ± 0.83	17	3.2 ± 0.33	0.82	0.39
14	19	4.6 ± 0.65	17	3.3 ± 0.19	0.88	0.34
13	18	4.7 ± 0.73	17	3.4 ± 0.31	0.94	0.26
12	19	4.6 ± 0.79	17	3.4 ± 0.29	0.94	0.26
11	19	4.2 ± 0.68	17	3.4 ± 0.33	0.96	0.11
10	18	4.0 ± 0.58	17	3.4 ± 0.35	0.94	0.05
9	19	3.9 ± 0.61	17	3.4 ± 0.41	0.90	-0.08
8	19	4.3 ± 0.68	17	3.5 ± 0.39	0.96	-0.10
7	19	5.1 ± 0.72	17	4.4 ± 0.52	0.91	-0.32
6	19	6.7 ± 1.04	17	6.3 ± 0.73	0.73	-0.53
5	19	9.5 ± 1.29	17	9.6 ± 1.17	0.51	-0.81
Percent of Total Variance Explained					**76.14**	**13.34**

** Slice 5 is located distally on the femoral shaft just above the condyle ridge; slice 15 is located proximally at the lower limit of the third trochanter The thickness of each slice is 5% of the overall bone length.* Principal component analysis (PCA) showed two axes of variation in bone areas. The principal axis of variation PC1 of a principal component analysis of marrow cavity area accounted for 76.1% of the variation in the data and reflected variation in overall size, all loadings having high and positive values. The second principal component PC2 reflected variation in cavity shape according to site and accounted for 13.3 % of the total variance. The component loadings in the distal half of the femoral shaft had negative component loadings and greater marrow cavity area while those of the proximal femur had positive component loadings and lower marrow cavity area.

Total cross sectional area (mm^2)

The overall total cross sectional areas of slices from the femoral shaft of OVX rats were significantly greater on repeated measures ANOVA ($p< 0.002$) than SHAM rats

(Table 3-1). There was also significant variation of the total cross sectional areas of individual slices along the length of the femoral shaft ($p < 0.0005$). However, the lack of any significant interaction between slice and treatment indicated that this pattern of variation did not differ significantly between the two treatment groups.

Table 3-4 gives the results of the PCA of the 15 slices that made up the femoral shaft. A total of two principal components were sufficient to explain 93% of the variation in the original data. The effect of treatment on each of the PC scores was investigated by one-way ANOVA. The PC1 scores of OVX rats were significantly higher than those of SHAM rats ($p<0.002$), i.e. they had larger overall total cross sectional areas than SHAM rats. PC2 scores showed no significant differences between treatments. Therefore, while the overall size of the shafts of rat femurs were greater in OVX rats compared to SHAM rats, their external shapes were unchanged.

Table 3-4. Means and SD's of the total cross sectional areas of consecutive slices from the femoral shafts of ovariectomized and sham operated rats and results of principal component analysis.

	Cross sectional area (mm^2)					
	Ovariectomized rats		Sham operated rats		Component loadings	
Slice	N	Mean ± SD	N	Mean ± SD	PC1	PC2
15	18	14.1 ± 1.39	17	13.0 ± 1.12	0.91	0.31
14	19	13.6 ± 1.26	17	12.4 ± 0.99	0.93	0.28
13	18	12.9 ± 1.22	17	11.7 ± 1.10	0.95	0.27
12	19	12.3 ± 1.16	17	11.3 ± 0.88	0.96	0.22
11	19	11.9 ± 1.06	17	10.9 ± 0.99	0.97	0.19
10	18	11.5 ± 1.00	17	10.6 ± 0.77	0.98	0.06
9	19	11.1 ± 1.07	17	10.3 ± 0.79	0.97	-0.03
8	19	11.3 ± 1.01	17	10.2 ± 0.73	0.97	-0.14
7	19	12.0 ± 1.07	17	10.8 ± 0.79	0.94	-0.28
6	19	13.4 ± 1.38	17	12.4 ± 0.82	0.77	-0.51
5	19	16.4 ± 1.55	17	15.8 ± 1.30	0.61	-0.70
Percent of Total Variance Explained					**83.07**	**10.75**

** Slice 5 is located distally on the femoral shaft just above the condyle ridge; slice 15 is located proximally at the lower limit of the third trochanter The thickness of each slice is 5% of the overall bone length.* Principal component analysis (PCA) showed two axes of variation in bone areas. The principal axis of variation PC1 of a principal component analysis of total femoral cross sectional area accounted for 83% of the variation with uniformly high component loadings of equal sign indicating that this axis represented variation in overall size of the femurs. The second principal component PC2, accounting for 10.7 % of the variation, showed a pattern of negative component loadings in the distal half and positive component loadings in the proximal half of the femoral shaft reflecting a lengthwise variation in overall shape.

3.3.2. Mineral content of femoral shaft

Duel energy X-ray Absorptiometry (DEXA)

The total projected bone areas on DEXA (BA) were significantly greater in ovariectomized rats (n = 19, 1.75 ± 0.023) than in sham operated rats (n = 18, 1.69 ± 0.013) on ANOVA ($p<0.035$). The SHAM rats had significantly greater overall femoral BMD compared to the OVX rats ($p<0.0005$), but there were no significant differences in femoral BMC between the two treatment groups.

Mineral composition

The effect of ovariectomy surgery and segment (location in the femoral shaft) on the mineral composition (relative to the amount of organic and inorganic material: g/g air dry bone mass) of the femoral shaft was investigated by 2-way ANOVA (Fig. 3-4). The mineral composition was significantly greater in SHAM rats compared to the OVX rats ($p<0.0005$) (Table 3-5). There were significant differences in mineral composition between segments, with proximal segment A having the lowest mineral content compared to segments B and C ($p<0.0005$), and segment B having a significantly lower mineral composition compared to C ($p<0.031$). The mineral composition of the three segments in the SHAM rats was not significantly different from the mineral composition of the three segments in the OVX rats; i.e. there was no significant interaction between treatment and segment. Therefore, the mineral composition did not vary between the different regions of the femoral shaft in relation to whether the rats were ovariectomized or sham-operated.

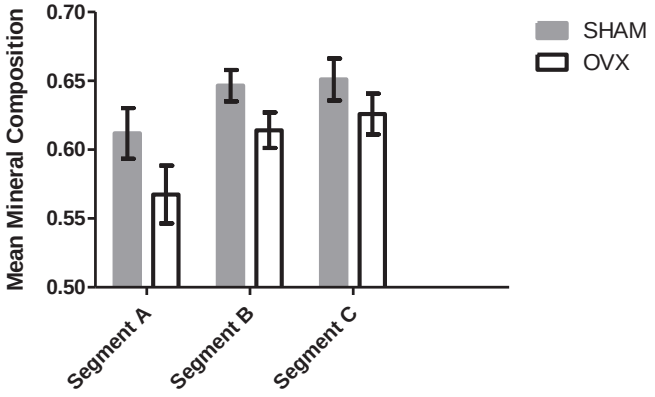


Figure 3-4. Mean mineral composition ± standard deviation (relative to the amount of organic and inorganic material: g/g air dry bone mass) of the two treatments sham-operated rats (SHAM) and ovariectomized rats (OVX), for the three segments of the femoral shaft.

Table 3-5. 2-way ANOVA, means, SD's of the mineral composition (relative to the amount of organic and inorganic material: g/g air dry bone mass) of the three segments of the femoral shaft from ovariectomized and sham operated rats.

	OVX		SHAM	
	N	Mean ± SD	N	Mean ± SD
Segment A	19	0.57 ± 0.02	17	0.61 ± 0.02
Segment B	19	0.61 ± 0.01	17	0.65 ± 0.01
Segment C	19	0.63 ± 0.02	17	0.65 ± 0.02
		df.	F-ratio	p-value
Treatment		1	120.884	0.0005
Section		2	82.116	0.0005
Treatment*Section		2, 102	0.226	0.798

** Segment A is located at the proximal shaft of the femur comprising of slices13-15, Segment B encompasses the midsection of the femoral shaft comprising of slices 9-12, and Segment C is located at the distal shaft comprising of slices 5-8.*

3.3.3. Biomechanical strength of the femoral shaft

Cross sectional moment of inertia (mm^2) (CSMI)

The overall CSMI of slices from the femoral shaft of OVX rats were significantly greater on repeated measures ANOVA ($p< 0.0010$) than SHAM rats (Table 3-1). There was also significant variation of the CSMI of individual slices along the length of the femoral shaft ($p< 0.0005$). However, the lack of any significant interaction between slice and treatment indicated that this pattern of variation did not differ significantly between the two treatment groups.

Table 3-6 gives the results of the PCA of the 15 slices that made up the femoral shaft. A total of two principal components were sufficient to explain 92% of the variation in the original data. The effect of treatment on each of the PC scores was investigated by one-way ANOVA. The PC1 scores of OVX rats were significantly higher ($p<0.008$) than SHAM rats, i.e. the OVX rats had a greater overall CSMI than SHAM rats. Similarly, a one-way ANOVA of PC2 scores had a significant effect on the variation between treatments ($p< 0.028$). The contrast scores between the upper and lower CSMI was significantly higher in the OVX rats than that of the SHAM rats.

This is in contrast to the finding from the ANOVA showing an overall increase with treatment, but showed no regional patterns with treatment.

Table 3-6. Means and SD's of the total cross sectional areas of consecutive slices from the femoral shafts of ovariectomized and sham operated rats and results of principal component analysis.

	Cross sectional moment of inertia (mm^2)					
	OVX		SHAM		Component loadings	
Slice	N	Mean ± SD	N	Mean ± SD	PC1	PC2
15	18	35.0 ± 6.56	17	32.3 ± 5.76	0.81	-0.50
14	19	31.7 ± 6.11	17	27.8 ± 5.14	0.87	-0.38
13	18	26.2 ± 4.69	17	22.6 ± 4.73	0.92	-0.33
12	19	23.2 ± 3.89	17	20.0 ± 3.29	0.96	-0.23
11	19	21.2 ± 3.53	16	18.4 ± 3.60	0.97	-0.15
10	18	19.7 ± 3.36	17	17.0 ± 2.49	0.98	0.03
9	19	19.0 ± 3.58	17	16.2 ± 2.40	0.97	0.13
8	19	19.1 ± 3.16	17	15.9 ± 2.32	0.96	0.26
7	19	20.8 ± 3.55	17	17.2 ± 2.48	0.93	0.35
6	19	24.3 ± 5.00	17	19.8 ± 2.50	0.76	0.52
5	19	31.3 ± 4.80	17	27.0 ± 3.20	0.80	0.34
Percent of Total Variance Explained					**81.67**	**10.45**

** Slice 5 is located distally on the femoral shaft just above the condyle ridge; slice 15 is located proximally at the lower limit of the third trochanter The thickness of each slice is 5% of the overall bone length.* The principal axis of variation (PC1) of a principal component analysis of total femoral cross sectional area accounted for 82% of the variation with uniformly high component loadings of equal sign indicating that this axis represented variation in overall size. The second principal component (PC2), accounting for 10.5 % of the variation, showed a pattern of negative component loadings in the proximal half and positive component loadings in the distal half of the femoral shaft reflecting a lengthwise variation in overall shape.

Compression strength

The effect of ovariectomy surgery and segment (location in the femoral shaft) on the extrinsic stiffness and yield strength of the femoral shaft was investigated by 2-way ANOVA (Table 3-7). There were no significant differences on two-way ANOVA between the extrinsic stiffness and segments in relation to treatment. Therefore, regardless of treatment or location, the overall structure of the segments of the femoral shaft displayed similar rigidity, i.e. they had similar ability to resist deformation to the overall bone structure before fracture.

There were no significant differences between the maximum loads needed to fracture the segments in relation to treatment. However, there were significant differences between segments and maximum load, where, proximal segment A and segment B (mid-shaft) were stronger than distal segment C ($p<0.0005$), i.e. there was a significant difference between segments in relation to the max load that could be sustained before fracture.

While not significant, there was evidence of a possible interaction between the maximum load and segments in relation to treatment ($p<0.077$). This suggests that the maximum load the segments could sustain before fracture may be influenced by region and whether the rats were ovariectomized or sham-operated.

Table 3-7. 2-way ANOVA, means and SD's of the extrinsic stiffness and maximum load of the three segments of the femoral shaft from ovariectomized and sham operated rats.

a) Extrinsic stiffness (N/mm)

	Ovariectomized		Sham	
	N	Mean ± SD	N	Mean ± SD
Segment A	16	6.9 ± 4.82	12	5.6 ± 2.42
Segment B	15	6.4 ± 3.53	12	6.2 ± 3.40
Segment C	15	7.3 ± 3.42	10	8.0 ± 2.73
		df.	F-ratio	p-value
Treatment		1	0.138	0.711
Section		2	1.311	0.276
Treatment*Section		2, 74	0.505	0.606

b) Maximum load (N)

	Ovariectomized		Sham	
	N	Mean ± SD	N	Mean ± SD
Segment A	15	959.9 ± 182.30	13	1102.8 ± 252.36
Segment B	14	990.3 ± 209.04	12	926.2 ± 178.59
Segment C	15	778.5 ± 183.08	13	706.8 ± 159.05
		df.	F-ratio	p-value
Treatment		1	0.003	0.957
Section		2	16.265	0.0005
Treatment*Section		2, 76	2.659	0.077

** Segment A is located at the proximal shaft of the femur comprising of slices13-15, Segment B encompasses the midsection of the femoral shaft comprising of slices 9-12, and Segment C is located at the distal shaft comprising of slices 5-8.*

3.4. Discussion

3.4.1. Morphological changes in the femoral shaft following ovariectomy or sham ovariectomy.

The study showed that there were significant differences in total area, marrow cavity area and bone area in the shaft of the femur in rats undergoing ovariectomy from those of rats undergoing sham ovariectomy.

The relative general increase in the mean total area of the slices from the femoral shaft in ovariectomized rats (which could be considered to represent the total volume of the femoral shaft) indicates that there is greater overall growth of periosteal bone following ovariectomy. The lack of any significant variation in the overall area of slices between regions indicates that ovariectomy uniformly augmented periosteal deposition of bone in the femoral shaft. These results fit in with the finding of an increase in projected bone area on DEXA, and with previous work that reported general additional periosteal and no additional endosteal deposition of bone within the femoral shafts in ovariectomized compared with non-ovariectomized rats (21-23). However, the effect of ovariectomy on periosteal apposition is reported to vary with age. Therefore, while significant postoperative periosteal bone growth is reported in the femoral shaft of 'mature' ovariectomized rats (>3 months in age), such growth is either markedly reduced, or absent in 'old' ovariectomized rats (>1 year in age) (7, 24, 25). Given that our rats were ovariectomized at 6 months of age, bone growth would have been continuing postoperatively (26) and may have been augmented by ovariectomy, whereas this effect may not be present in ovariectomized old rats (25).

The mean marrow cavity area of the slices from the femoral shaft, which could be considered to represent the total volume of the marrow cavity, was relatively enlarged in ovariectomized compared with that of sham ovariectomized rats, indicating that ovariectomy either generally accelerated endosteal bone absorption or withheld bone disposition in the femoral shaft. This result agrees with previous reports of augmented endosteal absorption following ovariectomy (7,23, 27). However, the finding that the proximal cavity tended to increase in size to a greater extent than the distal cavity following ovariectomy is new, and indicates that the oestrogenic effects on the dynamic

of endosteal bone disposition or re-absorption may vary between different regions of the femur. This finding contradicts what was suggested by Peng *et al* (7). In their work they hypothesized that endosteal resorption and periosteal apposition occurred in parallel as an explanation for a lack of change in bone area between SHAM and OVX rats. However, their conclusion was based on area calculation from only one site (the mid-shaft of the femur). Therefore, while that may have been true for that particular site, this study has shown that the pattern of change in bone area along the length of the femoral shaft differs between the two treatment groups. The OVX rats had greater distal bone area compared with that of SHAM operated rats, yet the bone area in the proximal region was relatively smaller for OVX rats compared to the SHAM rats. It is worthy of note that the total volume of bone in the shaft of the femur, as represented by overall change in the bone areas of all slices, did not differ between treatments. Therefore, there may have been some compensatory adjustment between regions in the rates of endosteal bone apposition during the phase of continuing growth, e.g. the diversion of bone deposition from proximal to distal sites. No information could be found in humans concerning regional variation in endosteal bone disposition, or re-absorption in the femoral shaft of osteoporotic bone.

3.4.2. Changes in mineralization of the femoral shaft following ovariectomy

Ovariectomy is known to result in a relative reduction of bone mineral density within the femur, and has been shown to result in increased porosity in the femoral shaft (28-30). The overall reduction in the mineral composition of the femoral shafts of ovariectomized rats, compared with that of sham ovariectomized rats, found in this study fits in with previously reported work. However, this study also found the mineral content within the femoral shaft varied, with the proximal segment (A) having significantly less mineral relative to the weight of the bone segment (amount of organic and inorganic material: g/g air dry bone mass). This variation did not appear to be related to whether the rats were ovariectomized or sham-operated. Therefore it could be suggested that the rate of mineral loss within the femoral shaft of ovariectomized rats was even. Previous work on mineral loss within the femurs of ovariectomized rats has reported uneven mineral depletion at different sites (31). However, in these cases the previous work cannot be strictly compared to the current work as the femur was divided up into slightly different regions. These earlier reports concluded that the reduction in

mineral content was greater in the distal metaphyseal region (25, 31) compared to the diaphyseal and proximal regions of the femur (31). The difference in results could be due to the previous studies dividing the femoral regions according to areas with high trabecular content versus sites with largely cortical bone sites. For the current study, the three segments investigated comprised of the dominant cortical bone region of the femoral shaft.

3.4.3. Changes in bone strength following ovariectomy

The mechanical competence of bone is determined by both the material and the geometry of the bone (32). Under normal conditions the shaft of the femur is subjected to bending forces when walking or standing, due to the slightly curved nature of the whole bone structure. Therefore, traditional methods for testing usually involve subjecting the whole femur to a bending test to determine bone strength. Using smaller segments to investigate the different regions of the femoral shaft did not allow for this type of testing so the predicted bending strength was calculated using CSMI, and the structural strength was determined using a direct axial compression of the different segments.

The impact of the resulting change in the structure of the bone on strength can be seen in the increased predicted bending strength of the femoral shaft of the OVX rats. This is in agreement with previous work where increased bone size typically follows ovariectomy. While CSMI is a calculated measure and not an actual determinant of strength, it has been shown that CSMI, along with the investigation of bone area and bone size changes, can successfully predict up to 70% - 80% of whole bone strength (8). The work of Hogan *et al* (2000) demonstrated not only an increase in CSMI at the mid-shaft of the femur, but a slight increase in the extrinsic properties of the femur, reflecting the effect of bone size on increased bone strength (14). However, in the current study the increased bending strength predicted by the CSMI was not reflected in the extrinsic properties of the femoral shaft. It may be that the compression method used in this study was not the most effective in demonstrating the effect of bone geometry on bone strength, nor was it effective in determining the material strength on the segments as it would have been expected to see some evidence of material weakening due to the loss of bone shown by the reduced BMD and mineral composition found in the OVX rats.

As far as regional strength testing is concerned it should not be ruled out. The principal component analysis did show a regional variation in CMSI, suggesting that predicted strength effects of ovariectomy were uneven along the shaft. Therefore, regional strength assessment may be of benefit alongside whole bone strength testing, although in small animal studies this does pose a problem, as mechanical testing becomes increasingly difficult as the bone samples get smaller.

3.4.4. Conclusion

The results of this study show that ovariectomy had differing effects on the morphology and predicted biomechanical characteristics of the proximal, middle and distal portions of the femoral shaft of ten month old rats. These effects are likely to result from differences in the rates of endosteal and periosteal change. The significant loss of BMD and mineral composition in the femoral shaft of OVX rats did not affect the compression strength of the three segments that made up the femoral shaft, possibly due to the method used. The direct sectioning method used in this study was sufficient to detect changes in bone geometry that are known to occur in the ovariectomized rat model.

3.5. References

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4. The effect of formulated goat milk on bone growth and mineral accretion and morphology in growing female rats.

The method developed in chapter 3 was able to detect changes in the morphological disposition of the femoral shaft in ovariectomized rats. The following chapter will use this method to investigate the effect of a goat milk based diet on mineral accretion in growing rats from 3 weeks of age until mature 5 month old rats. Results of Chapter 2 suggest that there may be benefit in a goat milk diet being based on whole milk rather than a combination of vegetable oils and skim milk. Therefore the composition of the goat milk diet in this trial was based on whole milk powder using skim milk powder to help balance the diet for protein and fat content.

Compromised diet group

The rats fed the non-milk diet in the trials described in Chapters 4 and 5, became compromised after a diet deficiency was discovered post ovariectomy surgery discussed in Chapter 5. There was a total of two hundred rats grown for these trials in which sixty were involved in Chapter 4; while the remaining one hundred and forty rats were grown alongside for the trial described in Chapter 5 (of which samples were collected for experiments described in Chapters 6 and 7).

There was a total of sixteen rat deaths associated with this deficiency and all occurring after ovariectomy surgery as described in Chapter 5. This initially disguised the cause of the deaths as pathology believed the deaths were from infection due to surgery. These events were compounded with a maintenance failure at the facility where the animals were housed. These combined events delayed the ability of the pathologists to ascertain the cause of the infections and subsequent deaths. The infection was treated with two courses of antibiotics, the first for six days and the second for ten days. The antibiotics were given to all the rats in the trial as it was believed that all the rats were potentially at risk. Eventually it became apparent that all the deaths were occurring in the rats fed the non-milk diet, and a diet deficiency was considered as a possible cause. Early investigation of the diet found that the level of Biotin was below the recommended amount and extra Biotin was added to the existing non-milk diet at 27 weeks of age. The amount given was 1mg/kg of diet to increase the biotin levels up to 2mg/kg of diet as suggested by the National Research Council (1). There was no significant improvement in the rats and animals continued to expire. During the investigation into the possible Biotin deficiency pathologists first raised the possibility that a vitamin A deficiency was the underlying cause of the infections and deaths (Fig. 5-1). Therefore the control diet made in June was tested for vitamin A. The results concluded that there was no vitamin A present in the diet. Next the supply of vitamin pre-mix that was made for the diets was tested for vitamin A content and none was found. New vitamin pre-mix was purchased and new diets made. The rats were put on the new diet at 32 weeks of age. The new control diet also contained the increased Biotin levels of 2mg/kg of diet. The newly purchased vitamin mix was tested for vitamin A and found to have 0.24 mg/kg of diet (700 IU/kg of diet); instead of the expected 0.86 mg/kg of diet (2,500 IU/kg); resulting in only 28% of the expected amount. It is uncertain as to whether this low level was due to a miscalculation when

the vitamin pre-mix was prepared or the storage methods used by the third party that made the mixture. Unfortunately the time taken to work through this entire process meant that the rats fed the non-milk diet suffered from a vitamin A deficiency throughout the trials.

The vitamin mix purchased by a third party did not have a sufficient amount of vitamin A to meet the minimum dietary requirements of laboratory rats. The rats fed the non-milk diet were the most affected by this as they had no other sources of vitamin A in their diet. The rats fed either of the milk diets were not affected as both goat and cow's milk provided adequate levels of vitamin A at 1.3 mg/kg of diet and 0.7 mg/kg of diet respectively. The rats fed the non-milk diet were originally designed to be control for the milk diet, the SHAM rats the control for ovariectomy surgery, the OVX rats the control for the OVX rats fed the milk diets, and the OVX ALD rats the control for the OVX ALD rats fed the milk diets. However, the vitamin A deficiency rendered the non-milk control diet ineffective and therefore was reassigned as a third diet (non-milk).

Vitamin A deficiency is known to cause abnormal bone growth due to increased remodelling in the epiphysis region, as well as continued periosteal expansion and the reduction in osteoclast numbers at the periosteal surface (2, 3). Therefore, it is expected that the results of the parameters tested for this diet group may be unexpected and could make drawing conclusions difficult. The only other option available in this situation was to remove the three rat groups fed the non-milk control diet from experimental analysis. It was decided to proceed with the first option and the control group became the third diet, a non-milk group.

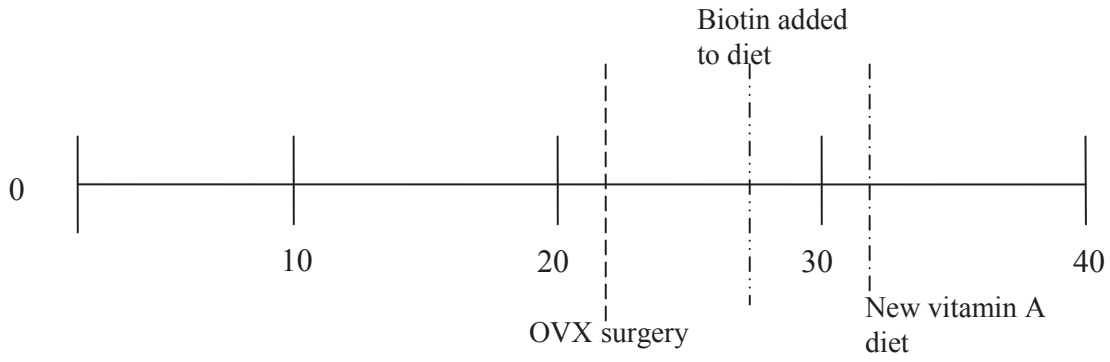


Figure 4-1. Timeline of diet changes in an attempt to correct suspected diet deficiencies.

Timeline represented as age of rats. Ovariectomy (OVX) surgery was conducted at 22 weeks of age. Extra Biotin was added at 28 weeks of age and a new diet with newly acquired vitamin mix was introduced at 32 weeks of age.

The diet containing the vitamin A deficiency was fed to the rats in the non-milk diet group from weaning (3 weeks of age). Previous research has reported deaths of rats after 11 weeks of being fed a vitamin A deficient diet (4, 5). The rats in the current trial survived for 20 + weeks before dying suggesting that they were receiving at least some vitamin A. This survival during growth may be due to the *ad libitum* access to their diet, therefore, giving them sufficient amounts to maintain minimal health. However, in preparation for the ovariectomy trial the rats had their diets restricted to 20g/rat/day. This restriction may have been sufficient to cause the animals to expire.

4.1. Introduction

Osteoporosis is a metabolic bone disease that is common in many developed countries. It is characterized by a reduction in bone density and in bone strength leading to pathological fracture. An important component of reducing the incidence of osteoporosis is prevention. It is widely accepted that peak bone mass is important in preventing the development of osteoporosis in old age. Therefore, if bone mass is increased to its optimal levels by the onset of menopause, then the inevitable bone loss that occurs with old age will not be so great as to increase the risk of fractures (6).

Bone mass peaks towards the end of the 2nd and within the 3rd decade in humans, although it is also accepted that different sites in the skeleton peak at different times (7, 8). There are a number of factors that influence 'Peak bone mass' including genetics, environment, exercise and nutrition (9). In terms of nutrition, dietary calcium optimises mineral accretion during bone growth which may also assist in achieving peak bone mass (7, 10). In the western world, milk is considered to provide the majority of essential dietary calcium. Considerable research in this area has led to the understanding that milk has the optimal ratio of calcium and phosphorus (2:1) for the enhanced absorption of minerals. This research has been largely directed towards dairy cow products, however, research has shown cow's milk products are not affordable by many of the poorer populations in third world countries (11-13). In these locations milk from goats provides a cheaper and more accessible supply of dairy food, and has been referred to as the "poor man's cow" (14). While goat milk research has received less attention, animal trials have shown increased mineral absorption in growing rats fed a goat's milk based diet compared to rats fed either a standard control diet or a cow's milk based diet (15, 16). Similar results have also been seen in a human trial, where investigations of goat milk supplemented diets in children have shown increased skeletal mineralization and bone density compared to children given cow's milk diets (17). Other human trials have also suggested that goat's milk offers similar benefits to cow's milk, although these studies focused their trial groups on undernourished children, or children with digestive malnutrition (12, 13).

The aim of this study was to investigate the effect of a goat milk based diet on bone growth and mineral accretion in growing rats until 5 months of age. Optimizing bone

mass during growth may increase peak bone mass to a level that may reduce the incidence or effects of osteoporosis later in life.

4.2. Methods and materials

4.2.1. Animals

Sixty Sprague-Dawley female rats aged 3 weeks (weanlings) were sourced from the Small Animal Production Unit (SAPU) at Massey University. They were kept individually caged in a temperature (22°C ± 2°C) and light (12 hour day/night cycle) controlled room at the same facility. In week one of the trial they were randomly allocated into three groups with 20 rats in each group. All rats were fed the non-milk diet for one week and then introduced to their group diets.

4.2.2. Diets

Rats were feed a semi-synthetic diet *ab libitum* with vitamin and minerals' content for growing rats based on the AIN93G diet plan (1). The diets were balanced to match the composition of the goat milk diet in calcium (0.69%), and phosphorus (0.59%) content; and protein was set at 20% (Table 4-1). The protein source was egg albumin for the non-milk diet, goat whole and skim milk for the goat diet, and cow whole and skim milk for the cow diet.

Table 4-1. Diet composition (%) for non-milk and experimental diet formulations.

	Treatment Group		
	Non-milk	Goat	Cow
Egg Albumin [1]	26.5		
Goat (whole + skim milk)[2]		63.8	
Cow (whole + skim milk)[3]			61.6
Starch	51	30	32
Soya oil	11		
Cellulose	5	5	5
Vitamin mix[4]	1	1	1
Mineral mix+ trace salts[5]	3.5		
Trace salts		0.2	0.2
Calcium Hydrogen phosphate ($CaHO_4P$)	2		0.2

[1] Non-milk diet using egg albumin as the protein source.
[2] Goat milk protein ratio 57:43 whole milk powder:skim milk powder.
[3] Cow milk protein ratio 67:33 whole milk powder: skim milk powder.
[4](mg kg^{-1} diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, $_{D}$-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, $_{D}$-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg kg^{-1} diet) ergocalciferol 25.0, cyanocobalamin 50.0.
[5](g kg^{-1} diet) calcium 0.69, chloride 7.79, magnesium 1.06, potassium 4.86, phosphate 0.59, sodium 1.97; (mg kg^{-1} diet) chromium 1.97, copper 10.7, iron 424.0, manganese 78.0, zinc 48.2; (μg kg^{-1} diet) colbalt 29.0, iodine 151.0, molybdenum 152.0, selenium 151.0.

4.2.3. Bone densitometry by Duel energy X-ray Absorptiometry (DEXA)

At 7, 13, and 19 weeks of age *in vivo* DEXA were performed to assess whole body, lumbar spine and femur bone density. The measurements were bone mineral density (BMD), bone mineral content (BMC), bone area, whole body - fat mass, lean mass, bone mass, and percent of body fat. Measurements were taken using a "Hologic Discovery A" bone densitometer (Bedford, MA, USA). On each day that scans were undertaken, a quality control (QC) scan was taken to ensure that its precision met the required DEXA manufacturer's coefficient of variation. The coefficient of variation (CV) for the QC data was 0.98% – 1.01%. Coefficient of variance for the femurs with repositioning between the scans was 1.20%, and without repositioning the scans the coefficient was 0.60%. These values ranged between 0.61% and 1.38% for the lumbar spine.

Each rat underwent three regional high-resolution scans of the whole body, spine, left and right femurs. Before scanning the rats were anaesthetised with a drug mixture of Ketamine, Acepromazine (ACP), sterile water, and Xylazine at a ratio of (5:2:2:1). The

dose was administered via intra-peritoneal injection at 0.05ml/100g of body weight. Rats were positioned supine with right angles between the spine and femur and between femur and tibia.

4.2.4. Euthanasia and sample collections

At 19 weeks of age the rats were euthanized post-DEXA scanning, by exsanguinations under anaesthesia. The anaesthetic was administered at a dose rate of 0.1ml/100g of bodyweight, using the same mixture and ratio as described in the above section (DEXA). Both hind legs of each rat were subsequently removed by simple dissection, and placed in phosphate buffered saline (PBS) at -20° Celsius pending further analysis.

4.2.5. Femurs

Left femurs were thawed, and their weight and length determined after any residual adherent soft tissue had been removed. The measured length of each femur was then divided into five segments marked at 25%, 45%, 65% and 85% of the total length. Each segment was then sectioned along the transverse lines with a diamond wheel saw. Subsequent measurements were taken from the three segments (25%-85%) that constituted the femoral shaft. These segments spanned 60% of the overall length, and extended from the upper limit of the intercondylar ridge (segment C) at the distal end of the femoral shaft, to the upper limit of the third trochanter (segment A) at the proximal end of the femoral shaft (Fig. 4-2).

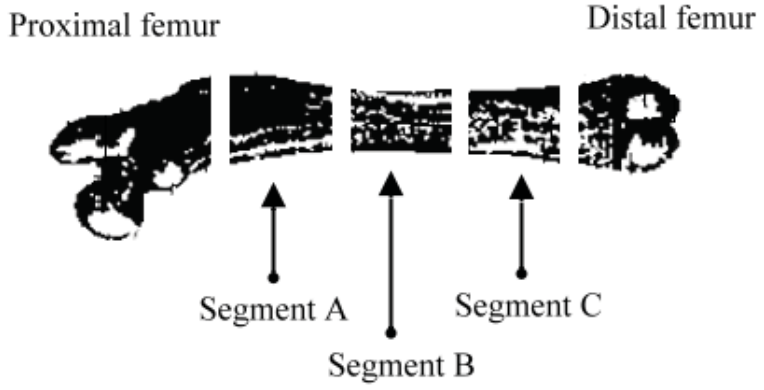


Figure 4-2. Picture describing the five sections cut from the entire femur, including the three segments that make up the femoral shaft used in this study. They are identified as Segment A (proximal shaft), Segment B (mid-shaft), and Segment C (distal shaft).

4.2.6. Imaging

The proximal and distal bone areas of the transverse cross sections of each segment were scanned with an Epson Perfection 3200 Photo colour scanner to a resolution of 4800 dpi. This gave a total of two scanned surfaces per segment and a total of six surfaces for each femoral shaft. The inner and outer edges of the bone profile of each slice were enhanced by image analysis software (Ulead Photoimpact 6 software, Ulead Systems, Inc). The bone area, total cross sectional area, and marrow cavity area (Fig. 4-3) of the enhanced upper and lower images of each slice were determined using an engineering software package (Image J, U. S. National Institutes of Health, Maryland, USA).

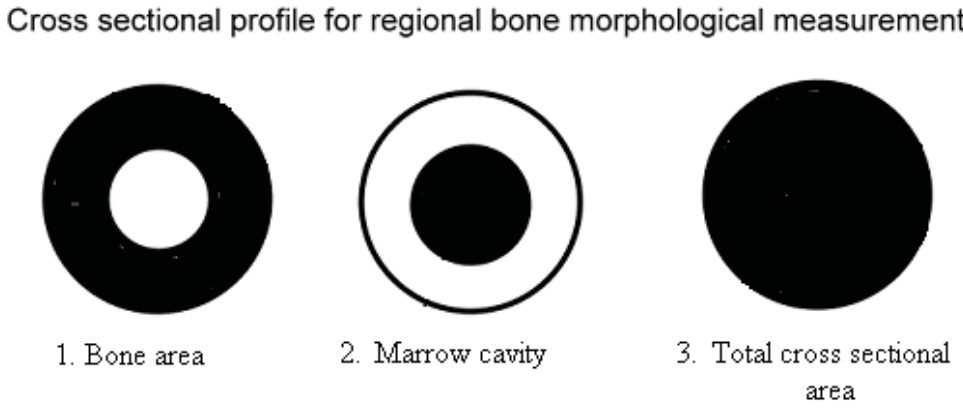


Figure 4-3. Picture describing the transverse cross sectional profile of the femur slice.

1. Shows the area used to calculate the bone area (mm^2).
2. Shows the area used to calculate marrow cavity area (mm^2).
3. Shows the area used to calculate the total cross sectional area (mm^2).

4.2.7. Ash content

The three segments of the femoral shaft were ashed at 660^{o}C. The mineral composition (relative to the amount of organic and inorganic material: g/g dry bone mass) of each segment was calculated by dividing the ash content (grams) by dry weight (grams).

4.2.8. Statistical analysis

Data were analyzed in the statistical package "SAS" (version 9.3) (Sas Institute, Cary, USA). DEXA, bodyweight and diet intake data were found for the most part to be normally distributed. Where non-normal data were found a log transformation was required to obtain near normal distribution on graphic analysis. The mean bone area,

mean marrow cavity area, mean total cross sectional area, and mineral composition (g/g per dry weight bone mass) of the three segments taken from the femoral shaft were each normally distributed and, therefore, were amenable to parametric analysis. Analysis was done by General linear model (GLM) two-way ANOVA and significance was set at ($p < 0.05$).

The effects of diet on mineral composition (g/g per dry weight bone mass), bone area (mm^2), marrow cavity area (mm^2) and total cross sectional area (mm^2) in the femoral shaft were compared by one-way repeated measures ANOVA. The pattern of variation of each of these parameters between the three segments that made up the femoral shaft was explored using multivariate principal component analysis (PCA). A one-way ANOVA was then conducted on the individual principal component scores of each axis of variation in order to statistically compare the effects of the diets.

The data from bone samples damaged during preparation (i.e. broken during cutting) were removed from the analysis.

4.3. Results

Two rats died during the trial due to adverse reactions to the anaesthetic. The diet compositions including calcium, phosphorus, percent fat and percent protein were confirmed by chemical analysis (data not shown). Weekly diet intake showed no significant difference between groups throughout the trial (data not shown).

4.3.1. Duel energy X-ray Absorptiometry (DEXA)

Bodyweight and Whole body composition

Weight gain by all the rats was noted during the trial, and bodyweight between the groups did not differ during the first 4 weeks (Fig. 4-4). However, at 13 weeks of age, the rats fed the non-milk diet showed a significantly reduced gain in bodyweight, compared to the rats fed the cow's milk diet ($p<0.0006$) and the rats fed the goat's milk diet ($p<0.0011$). At the end of the trial the rats fed the cow's milk diets and goat's milk diets had significantly greater bodyweight compared to the rats fed the non-milk diet ($p<0.0001$).

At 7 weeks of age there were no significant differences between the three rat groups. At 13 weeks of age the rats from the non-milk group were significantly lower in whole body BMC than the rats fed the cow's milk diet ($p<0.012$). At the end of the trial (19 weeks of age) the rats fed the non-milk diet had less whole body BMC ($p<0.0435$), lean mass ($p<0.0317$) and bone mass ($p<0.0025$) than the rats fed the cow's milk diet.

There were significant increases within each group in whole body area, BMC and BMD ($p<0.0001$) over the course of the trial (Table 4-2). There were also differences between diets for whole body area ($p<0.0199$) and whole body BMC ($p<0.0135$). However, post-hoc testing did not reveal where these differences were.

Table 4-2. Repeated measures ANOVAs of Whole body area (cm^2), bone mineral content (BMC)(g), and bone mineral density (BMD) (g/cm^2), fat (g), lean (g), bone mass (g) and percentage fat (pfat).

Whole body area (cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	4.23	**0.0199**
Week	1,51	207.95	**0.0001**
Week* Diet	2,51	0.84	0.4393
While body BMC (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	4.69	**0.0135**
Week	1,51	544.76	**0.0001**
Week * Diet	2,51	0.47	0.6287
While body BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	2.15	0.1272
Week	1,51	375.71	**0.0001**
Week * Diet	2,51	4.51	**0.0158**
While body fat (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	1.23	0.3013
Week	1,51	0.40	0.5304
Week * Diet	2,51	3.60	**0.0345**
While body lean (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	1.34	0.2715
Week	1,51	11.68	**0.0012**
Week * Diet	2,51	3.39	**0.0415**
While body bone mass (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	1.04	0.3600
Week	1,51	1.10	0.3003
Week * Diet	2,51	4.94	**0.0110**
While body pfat (%)			
Factor	df.	F- statistic	P- value
Diet	2,51	0.29	0.7487
Week	1,51	0.27	0.6037
Week * Diet	2,51	2.38	0.1028

Lumbar spine

At 13 weeks of age the rats fed the cow's milk diet had significantly greater lumbar spine BMC ($p<0.0023$) and BMD ($p<0.0073$) than the rats fed the non-milk diet (Fig 4-5). At the same age the rats fed either of the milk diets had significantly greater area ($p<0.0104$) than the rats fed the non-milk diet. By the end of the trial (19 weeks of age) the rats fed the non-milk diet had significantly smaller lumbar spine areas ($p<0.0021$), less BMC ($p<0.003$) and BMD ($p<0.0157$) than the rats fed the cow's milk diet (Fig. 4-4).

There were significant increases within each group in lumbar spine area, BMC and BMD ($p<0.0001$) over the course of the trial (Table 4-3). There were also differences between diets for lumbar spine area ($p<0.0018$), BMC ($p<0.0041$) and BMD ($p<0.0082$). Post-hoc testing revealed significantly greater increases over the period from 7 to 13 weeks of age for lumbar spine area ($p<0.0163$), BMC ($p<0.0464$), and BMD ($p<0.0464$), in the rats fed the cow's milk diet compared to the rats fed the non-milk diet. Similarly between 7 to 19 weeks of age the rats fed the cow's milk diet had greater increases in lumbar spine area ($p<0.0209$) and BMC ($p<0.0363$) compared to the rats fed the non-milk diet.

Table 4-3. Repeated measures ANOVAs of Lumbar spine bone area (cm^2), bone mineral content (BMC) (g), and bone mineral density (BMD) (g/cm^2).

Lumbar spine area (cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	7.19	**0.0018**
Week	1,51	84.82	**0.0001**
Week* Diet	2,51	1.23	0.3000
Lumbar spine BMC (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	6.11	**0.0041**
Week	1,51	301.64	**0.0001**
Week* Diet	2,51	1.58	0.2152
Lumbar spine BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	5.28	**0.0082**
Week	1,51	228.84	0.0001
Week* Diet	2,51	1.21	0.3076

Femur

There were no significant differences between the rats fed the goat's milk diet and the rats fed the non-milk diet in femur BMD, BMC and area at 7, 13 and 19 weeks of age (Fig. 4-6). The rats fed the cow's milk diet had significantly greater BMD than the rats fed the non-milk diet at 19 weeks of age ($p<0.014$).

There were significant increases within each group in femur area, BMC and BMD ($p<0.0001$) over the course of the trial (Table 4-4). There was significantly greater BMD ($p<0.0438$) for the rats fed the cow's milk diet compared to the rats fed the non-milk diet between 7 and 19 weeks of age.

Table 4-4. Repeated measures ANOVAs of Femur bone area (cm^2), bone mineral content (BMC)(g), and bone mineral density (BMD) (g/cm^2).

Femur area (cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	0.32	0.7282
Week	1,51	161.69	**0.0001**
Week* Diet	2,51	0.03	0.9704
Femur BMC (g)			
Factor	df.	F- statistic	P- value
Diet	2,51	2.31	0.1093
Week	1,51	360.55	**0.0001**
Week* Diet	2,51	1.24	0.2987
Femur BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Diet	2,51	4.63	**0.0141**
Week	1,51	162.80	**0.0001**
Week* Diet	2,51	2.15	0.1269

4.3.2. Mineral composition

The overall mineral composition of the three segments of the femoral shaft was significantly different between diets on repeated measures ANOVA ($p<0.0005$) (Table 4-5b). There were also significant differences in mineral composition in the three segments of the femoral shaft ($p<0.0005$). However, the lack of any significant interaction between the segments and diets indicated that this pattern of variation did not differ significantly between the diets.

Principal component analysis (PCA) was then used to further examine the mineral composition of the shaft. Two PC's were sufficient to explain 79% of the total variation in the original data (Table 4-6). The effect of treatment on each of the PC scores was investigated by one-way ANOVA. PC1 scores showed a significant difference in mineral composition throughout the femoral shaft ($p<0.0005$). Post-hoc testing of PC1 scores showed that the rats fed the non-milk diet had significantly less overall mineral in the femoral shaft than the rats fed the cow's milk diet ($p<0.0005$) and the goat milk diet ($p<0.001$). The pattern of variation found in PC2 was not a result of the diets given to rats. This reinforces the results of the repeated measures where the rats fed either of the milk diets had significantly more overall mineral composition than the rats fed the non-milk diet; but these differences were not regional within the femoral shaft.

Table 4-5. Means, SDs, and repeated measures ANOVAs of overall bone area (mm^2), marrow cavity area (mm^2), total cross sectional area (mm^2), and mineral composition (g/g per dry weight bone mass) of segments of the femoral shaft from the three diets groups.

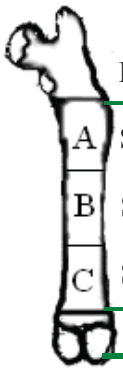
a)

Parameter	Non-milk		Cow		Goat	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Bone area (mm^2)	17	43 ± 3.2	19	46 ± 3.4	14	44 ± 3.5
Marrow cavity (mm^2)	16	28 ± 4.8	17	25 ± 3.6	13	25 ± 3.2
Total cross sectional area (mm^2)	17	71 ± 7.2	17	72 ± 5.7	16	68 ± 6.1
Mineral composition (g/g dry weight bone mass)	13	2.1 ± 0.06	17	2.2 ± 0.04	15	2.2 ± 0.05

b)

Bone area (mm^2)			
Between subjects	df.	F- statistic	P- value
Diet	2, 47	4.737	**0.013**
Within subjects			
Section	5	280.407	**0.0005**
Section*Diet	10, 235	4.817	**0.0005**
Marrow cavity area (mm^2)			
Between subjects	df.	F- statistic	P- value
Diet	2, 43	3.495	**0.039**
Within subjects			
Section	5	336.496	**0.0005**
Section*Diet	10, 215	2.129	**0.024**
Total cross sectional area (mm^2)			
Between subjects	df.	F- statistic	P- value
Diet	2, 47	1.027	0.366
Within subjects			
Section	5	335.014	**0.0005**
Section*Diet	10, 235	1.655	0.092
Mineral composition (g/g per dry weight bone mass)			
Between subjects	df.	F- statistic	P- value
Diet	2, 42	13.874	**0.0005**
Within subjects			
Section	2	108.624	**0.0005**
Section*Diet	4, 84	0.727	0.560

Table 4-6. Table of mineral composition (g/g per dry weight bone mass).



Ash weight / Dry weight	Mineral composition (g/g per dry weight bone mass)						Component loadings	
	Non-milk		Cow		Goat			
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	PC1	PC2
Segment A	17	0.73 ± 0.013	19	0.75 ± 0.023	17	0.76 ± 0.028	0.441	-0.863
Segment B	17	0.71 ± 0.016	18	0.73 ± 0.028	17	0.73 ± 0.027	0.822	0.041
Segment C	17	0.63 ± 0.043	19	0.67 ± 0.036	17	0.67 ± 0.039	0.728	0.477
Percent of Total Variance Explained							**46.70**	**32.48**

Component loadings from a principal component analysis, means and standard deviations (SD), for the three segments of the femoral shafts for the rats fed the non-milk, goat and cow's milk diets. PCA of the dry tissue mass and ash weight showed two patterns of variation. The first principal component, axis (PC1) accounted for 46.7% of the variance. The second principal component, axis (PC2) accounted for 32.5% of the variance. All component weightings of PC1 had large positive values, indicating variation in overall mineral composition. The second axis of variation (PC2) has a high negative value for proximal segment A, which contrasts with a somewhat high positive component loading for distal segment C. Section A is located at the proximal shaft of the femur; section B around the midsection of the femur, and section C is located at the distal shaft.

4.3.3. Morphology of the femoral shaft

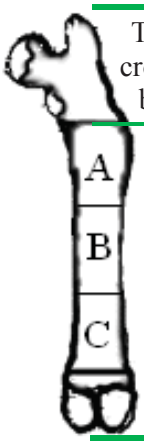
Bone area (mm^2)

The overall bone area of the segments from the femoral shaft was significantly different in repeated measures ANOVA between diets ($p<0.013$) (Table 4-5b). There were also significant differences between segments from different locations ($p<0.0005$). Further, the pattern of variation between regions differed significantly between diets ($p<0.0005$). Therefore diet influenced the overall bone area in the femoral shaft and significantly influenced the pattern of distribution of bone area along the femoral shaft.

PCA indicated where in the shaft those differences in bone area occurred, and where there was a diet effect. A total of two PC's were sufficient to explain 77% of the variation in the original data (Table 4-7). The effect on treatment on each of the PC scores was investigated by one-way ANOVA. PC1 scores of the rats fed the cow's milk diet had had an overall larger bone area throughout the femoral shaft compared to the rats fed the non-milk diet ($p<0.039$). Also, PC2 showed that the bone areas in the proximal half of the femurs of non-milk diet fed rats were relatively lower than those of

the cow diet rats, while the bone areas in the distal half of the shaft tended to be similar to that of the rats fed the non-milk diet ($p<0.004$). This reinforces the results from the repeated measures where the rats fed the cow's milk diet had a significantly greater overall bone area, and that there was a regional effect. i.e. that the rats fed cow's milk laid down bone at a significantly faster rate in the proximal region (segment A), yet was similar in bone area to the rats fed the non-milk diet in the distal region (segment C).

Table 4-7. Table of for bone area (mm^2)



Transverse cross section bone area	Bone area (mm^2) Non-milk N	Non-milk Mean ± SD	Cow N	Cow Mean ± SD	Goat N	Goat Mean ± SD	Component loadings PC1	Component loadings PC2
A Prox	18	8.8 ± 0.94	20	10.1 ± 0.93	16	9.6 ± 0.91	0.582	0.757
A Distal	19	7.4 ± 0.70	20	8.0 ± 0.86	18	7.3 ± 0.65	0.875	0.112
B Prox	19	7.2 ± 0.64	20	8.4 ± 1.04	18	7.8 ± 0.73	0.878	0.032
B Distal	19	6.2 ± 0.55	20	6.5 ± 0.04	18	6.3 ± 0.62	0.896	-0.183
C Prox	18	6.2 ± 0.46	19	6.4 ± 0.71	17	6.3 ± 0.43	0.737	-0.210
C Distal	18	6.6 ± 0.59	19	6.7 ± 0.54	17	6.5 ± 0.71	0.782	-0.317
Percent of Total Variance Explained							**63.89**	**12.75**

Component loadings from a principal component analysis, means and standard deviations (SD), for the three segments of the femoral shafts for the rats fed the non-milk, goat and cow's milk diets. PCA of the transverse proximal and distal bone surface areas of the three segments of the shaft showed two patterns of variation. The first principal component PC1 accounted for 63.9% and PC2 accounted for 12.8% of the variation. All component weightings of PC1 had large positive values, indicating variation in overall size. The pattern of component loadings indicated that PC2 reflected a contrast between the transverse bone areas of the proximal region segment (A) and the distal region of the shaft (segments B and C) i.e. a difference in the pattern of distribution of bone volume along the femoral shaft.

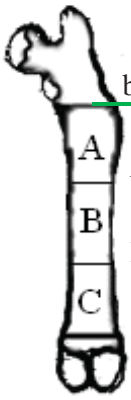
Total cross sectional area (mm^2)

There was no significant difference in overall total cross sectional area of the segments from the femoral shaft on repeated measures ANOVA between diets (Table 4-5b). There were significant differences between segments from different locations ($p<0.0005$). However, the lack of any significant interaction between the segments and diets indicated that this pattern of variation did not differ significantly between the three diet groups.

PCA of the transverse proximal and distal bone surface areas of the three segments of the shaft showed two patterns of variation. A total of two PC's were sufficient to

explain 78% of the variation in the original data (Table 4-8). The effect of treatment on each of the PC scores was investigated by one-way ANOVA. PC1 scores showed no significant difference between diet, whereas, the PC2 scores of the rats fed the cow's milk diet had lower PC2 scores than the rats fed the non-milk diet ($p<0.035$). This suggests that the total cross sectional areas of the rats fed the non-milk diet increased in the distal region (segment C) and the total cross sectional areas decreased in the proximal section relative to the rats fed the cow's milk diet.

Table 4-8. Table of for Total cross sectional area (mm^2)



Transverse cross section bone area	Total cross sectional area (mm^2) Non-milk N	Non-milk Mean ± SD	Cow N	Cow Mean ± SD	Goat N	Goat Mean ± SD	Component loadings PC1	Component loadings PC2
A Prox	18	16.1 ± 1.98	20	16.0 ± 1.53	18	15.7 ± 1.68	0.697	-0.284
A Distal	19	10.5 ± 1.08	20	11.1 ± 1.18	18	10.4 ± 0.93	0.905	-0.300
B Prox	19	10.7 ± 1.11	20	11.4 ± 1.35	18	10.8 ± 1.01	0.854	-0.297
B Distal	19	10.2 ± 1.11	20	10.0 ± 1.25	18	9.6 ± 1.03	0.907	0.052
C Prox	18	10.0 ± 1.10	19	9.9 ± 1.16	17	9.6 ± 1.01	0.782	0.323
C Distal	18	13.3 ± 1.78	18	12.7 ± 1.68	17	12.0 ± 1.53	0.740	0.572
Percent of Total Variance Explained							**66.92**	**11.58**

Component loadings from a principal component analysis, means and standard deviations (SD), for the three segments of the femoral shafts for the rats fed the non-milk, goat and cow's milk diets.PCA of the transverse proximal and distal bone surface areas of the three segments of the shaft showed two patterns of variation. The first principal component PC1 accounted for 66.9% and PC2 accounted for 11.6% of the variation. Combined these two components explained 78% of the total variation in the data. All component weightings of PC1had large positive values, indicating variation in overall size. The pattern of component loadings indicated that PC2 reflected a contrast between the total cross sectional area of the proximal region and the distal region of the shaft (segment C) i.e. a difference in the pattern of overall shape along the femoral shaft.

Marrow cavity area (mm^2)

The overall marrow cavity area of the segments from the femoral shaft was significantly greater on repeated measures ANOVA between diets ($p<0.039$) (Table 4-5b). There were also significant differences between segments from different locations ($p<0.0005$). Further, the pattern of variation between regions differed significantly between diets ($p<0.024$). Therefore, the overall marrow cavity area of the femoral shaft differed between diets and the diets also significantly influenced the pattern of variation within the segments along the femoral shaft. However the principal component analysis was

unsuccessful in showing differences the marrow cavity area between the groups. There were no significant differences between diets in femur length or dry weight of the femoral shaft (Table 4-9).

Table 4-9. Femur length and dry weight

Femur	Non-milk		Cow		Goat		p value
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	
Length (mm)	19	32.6 ± 0.20	20	33.1 ± 0.18	18	32.7 ± 0.21	0.240
Dry weight of femoral shaft (g)	17	0.27 ± 0.027	18	0.28 ± 0.026	16	0.26 ± 0.024	0.176

Means, standard deviations (SD) and one way ANOVA of total femur length and the overall dry weight of the three segments that make up the femoral shaft for rats fed the non-milk, goat and cow diets.

4.4. Discussion

A diet deficiency in the non-milk group was discovered post current trial that may have affected the outcomes analyzed in this experiment. The rats in this study had *ab-libitum* access to their diet, therefore it is unknown what amount of vitamin A they were able to consume. Consequently it cannot be said with any certainty the degree that they were affected by the deficiency. The effects of the deficiency on the measurements taken on the non-milk group are discussed below.

4.4.1. Effect of diet deficiency

The degree of impact of the vitamin A deficiency in the growing rats fed the non-milk diet is unknown. Comparisons of the DEXA BMC and BMD with similar work done by Kruger *et al* (2008) give mixed results. DEXA scans from 8 weeks of age show that the rats from the Kruger *et al* (2008) trial had greater lumbar spine BMD and BMC compared to the rats fed the vitamin A deficient diet of a similar age. There was no difference between the two trials in lumbar spine BMD or BMC at 13 weeks of age (15). However, by 19 weeks of age the rats fed the vitamin A deficient diet had greater lumbar spine BMD and BMC compared to the control rats in the trial conducted by Kruger *et al* (2008) (15). The rats fed the vitamin A deficient diet had lower femur BMD and BMC at 8 and 13 weeks of age compared to the rats fed the control diet reported in Kruger *et al* (2008); yet by 19 weeks of age there was no difference in BMD or BMC between either trial (15). Caution should be taken when interpreting the results in the current trial, even though the growing rats from Kruger *et al* (2008) and the rats fed the deficient diet had similar lumbar spine and femur BMD and BMC by 19 weeks of age. The vitamin A deficiency could have affected bone and other organs in ways not specifically measured in these studies.

4.4.2. Bodyweight and Whole body composition

This study investigated the effects of three semi-synthetic diets containing either goat milk or cow's milk or egg albumin, on bone growth and mineral accretion in female rats until five months of age. The two groups of rats fed the milk diets appeared to benefit with greater bodyweight and larger body size compared to the rats fed the non-milk diet. The reasons for this difference could not be attributed to food intake, nor were the compositions of the diets a factor. Therefore it was most likely due to the reduced

weight gain known to occur in animals that consume a diet deficient in vitamin A (18) The body composition results from the current study suggested that the rats fed the cow's milk diet had much greater whole body bone mass and BMC because of greater overall bone growth. Similar findings have been reported by Weaver *et al.* (2009) when investigating the effect of milk as a calcium source versus calcium carbonate on peak bone mass in female rats (19). They found that rats fed a cow's milk based diet had greater weight gain compared to the rats on a non-milk diet and that the weight gains were found primarily in BMC, BMD, and bone area (19). In the current study post-hoc testing did not find any significant difference between the rats fed the goat milk diet compared to the rats fed the non-milk diet in whole body measurements, despite the significant difference in bodyweight.

4.4.3. Morphological changes

Examination of bone area within the femoral shaft found that the rats fed the cow's milk diet had significantly greater overall bone area than the rats fed the non-milk diet. This along with the higher BMD suggested that there was rapid bone growth at the endosteal surface in the mineral rich environment provided by the cow's milk. This expansion at the endosteal surface was even more apparent in the proximal femoral shaft, where the rate of increase in bone area was more significant (segment A). The connection between milk and bone expansion at these surfaces is not a new concept. The addition of milk into a diet has been shown to result in bone expansion in humans. One study in adolescent children showed that cow's milk supplemented diets increased bone growth at both the endosteal and periosteal surfaces compared to children who's diets were not supplemented with milk (20). In a longer trial lasting over a seven year period, adolescent and late adolescent females were given either a placebo pill, a calcium supplement in pill form or cow's milk. Results showed that the consumption of calcium supplements either as the pill or milk, lead to a higher peak bone mass. The trial also concluded that the consumption of cow's milk had the added benefit of increased bone growth and also periosteal bone expansion (21). This would suggest that the extra benefits for bone that seem to accompany milk supplemented diets may be due to not only calcium but the presence and combinations of bioactive components, proteins and other minerals (21, 22).

The rats fed the goat milk diet also had significantly greater femur mineral composition (of the femoral shaft) than the rats fed the non-milk diet. Although this differences did not result in larger bone areas in the femur, these findings are in agreement with Kruger *et al* (2008), who reported that a formulated goat milk diet increased peak bone mass (observed using DEXA) in female rats compared to a non-dairy control diet (15). Similarly, DEXA showed increased BMD and BMC in humans given a daily cow's milk supplement compared to a group that continued their habitual diet over an 18 month period (23). Only one human trial could be found investigating the bone health effects of goat milk. Mack (1952) compared the effects of a supplemented goat milk diet and a supplemented cow's milk diet in healthy children aged from 6 to 13 years (17). However, they did point out a limitation in their investigation in that the trial was only five months long. Therefore, the authors suggested that while a trend did appear, caution should be taken when interpreting the results. They concluded that both groups had positive body growth and that there was no significant difference in skeletal maturity, although the children who drank goat's milk had increased bone density (17).

The lumbar spine was another of the bone sites investigated for the effects of diet on bone growth and mineral accretion. The rats fed the cow's milk diet had significantly greater lumbar spine BMD, BMC, and bone area. Further investigation showed that the rats fed the cow's milk diet showed a tendency to increase their BMC ($p<0.0565$) at a greater rate over the period of the trial than the rats fed the goat's milk diet. This tendency, however, did not equate to significant differences by the end of the trial.

Comparisons between the rats fed the goat milk diet and rats fed the cow's milk diet on mineral accretion in the current trial do not correspond with previously published results. In previous trials all showed a significantly higher mineral accretion in the rats fed the goat milk diets compared to the rats fed the cow's milk diets (15, 16, 24, 25). Lopez Aliaga *et al* (2000) concluded that the calcium content in the femurs in rats was greater in the goat milk diet compared to cow's milk and the non-milk diets (16). Similarly, Campos *et al* (2003) found increased calcium content in femurs of the goat milk group compared to either the cow or non-milk diets, and increased phosphorus content in both milk groups compared to the non-milk diet (25). Barrionuevo *et al* (2003) also observed higher zinc, copper and selenium contents in the femurs of growing rats fed a goat milk diet compared to those rats on the cow or non-milk diets

(24). These trials have all suggested that the greater mineral levels in the femur could be attributed to the greater absorbency of goat milk due to the higher percentage of the more readily absorbed medium chain fatty acids (15, 16, 24, 25). However, in the case of the present trial this greater absorption was not seen, at least in relation to retained bone mineral, nor were any differences found in bodyweight, body fat, or lean body mass. However, it should be pointed out that due to the different methods used in determining mineral content, direct comparison in mineral retention between the current study and previous work could not be made. The previous trials did not report overall ashed mineral content in their studies, only specific mineral contents such as calcium, whereas, in the current trial only DEXA BMC of the whole femur was reported. As for the two methods used, previous work has shown that ashing bone samples and DEXA BMC are highly correlated and therefore, at least general comparison between the two methods can be made (26). This study leaves many questions unanswered and further work is required.

4.4.4. Conclusion

The rats fed the goat and cow's milk diets surpassed the rats fed the non-milk diet in femur mineral composition, and had larger bodyweight. Mineral retention in the rats fed the goat milk diet is similar to that of the rats fed the cow's milk diet in the whole body, lumbar spine, and the femur parameters measured.

It is hard to draw any conclusions from this work as the effects of the vitamin A deficiency could have skewed the results. It is difficult to say whether either of the milk diets improved peak bone mass, thereby reducing the chances of osteoporosis like conditions in mature rats, as has been shown in previous work. However, with this in mind, the goat's milk diet was comparable in its effects to the cow's milk diet on the parameters investigated in the growing rat.

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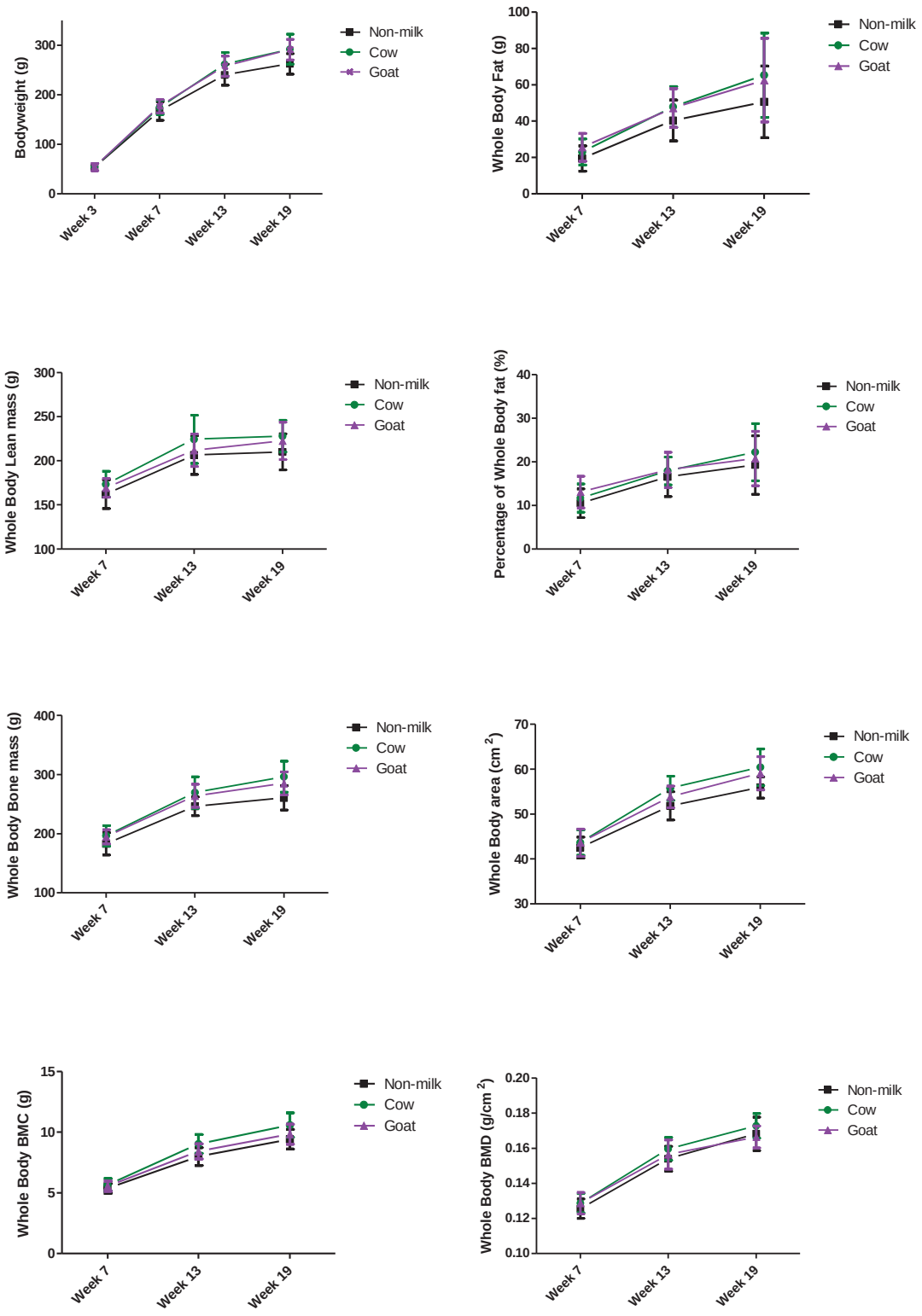

Figure 4-4. Graphs showing means and SDs for rat bodyweights, and the seven DEXA whole body composition measurements taken at 7, 13 and 19 weeks of age of the three diet groups. Non-milk (n=19), Goat (n=18), Cow (n=20).

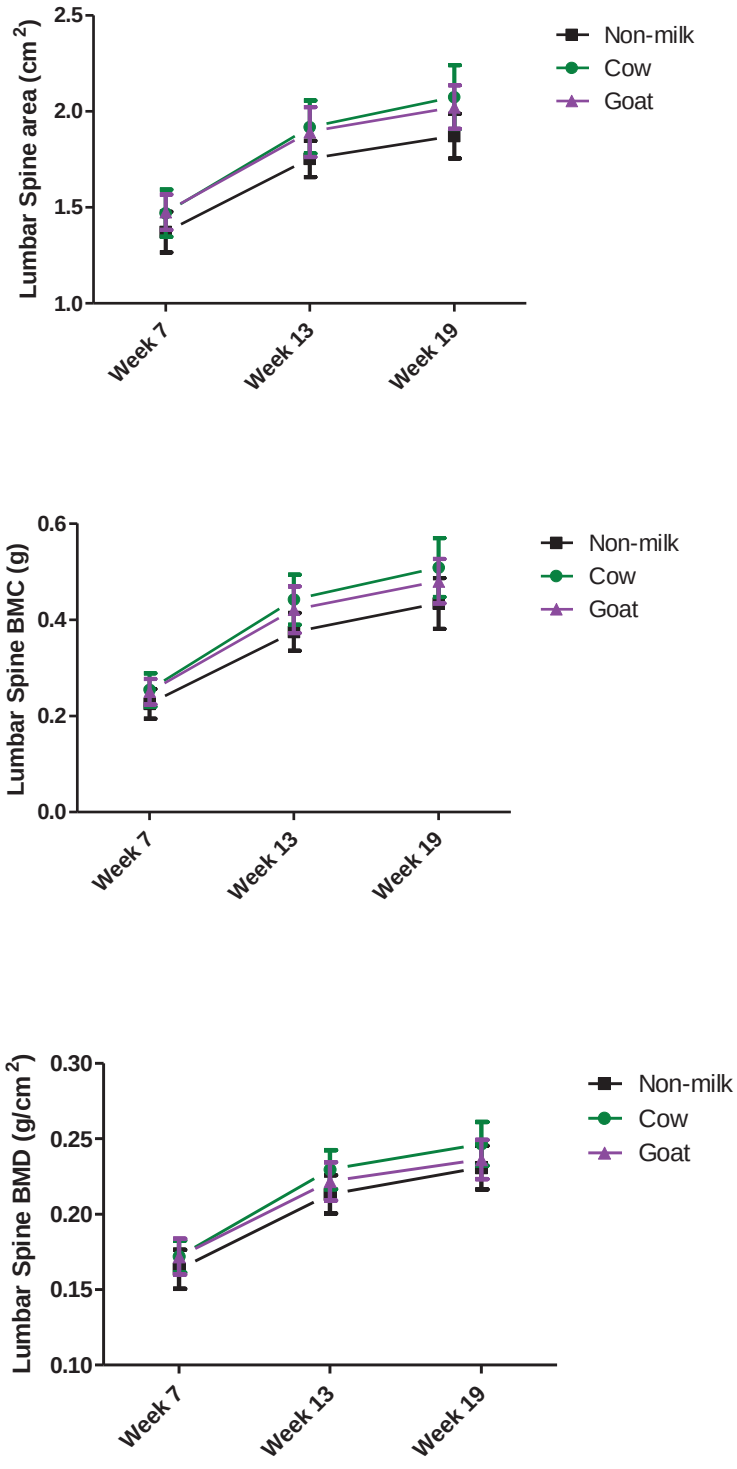

Figure 4-5. Graphs showing means and SDs for Lumbar Spine area, BMC, and BMD measurements from DEXA scan taken at 7,13, and 19 weeks of age. Non-milk (n=19), Goat (n=18), Cow (n=20).

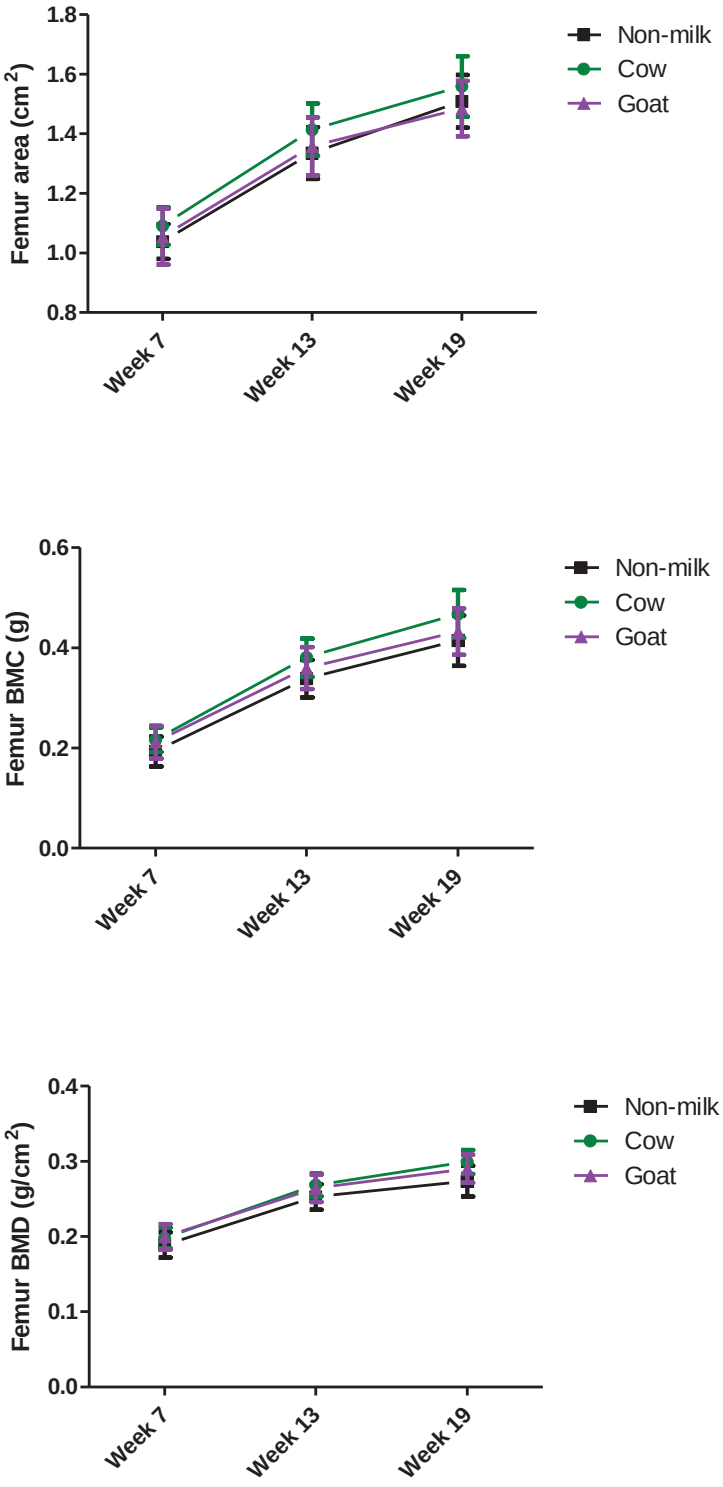

Figure 4-6. Graphs showing means and SDs for Femur area, BMC, and BMD measurements from DEXA scans taken at 7,13, and 19 weeks of age. Non-milk (n=19), Goat (n=18), Cow (n=20).

5. Effect of formulated goat milk and cow's milk diets with and without sodium alendronate on bone mass and regional bone morphological changes in the ovariectomized rat.

This study is linked to chapter 4 and tests the hypothesis that optimizing peak bone mass by long term consumption of a goat milk based diet may assist in reducing overall bone loss after ovariectomy in female rats. This study also compares the effect of the goat milk diet with and without the administration of the anti-resorption drug Alendronate in ovariectomized mature female rats.

5.1. Introduction

The composition of milk and its bioactive components are believed to enhance calcium absorption and retention. Subsequently milk has been recommended as the primary source of dietary calcium (1). A number of studies have shown increases in both bone mass and bone area after including milk or other calcium sources into the diets of children and adolescents (2, 3). However, it has also been shown that bone mass and bone area increases are transient, with the effects dissipating after the milk or other calcium supplements are removed (4, 5). In trials investigating the long term consumption of milk throughout life have shown increased peak bone mass, and a reduction in the incidence of bone fracture in aging women (6-10). During such investigations into the lifetime consumption of milk, it was also noted that the regular intake of milk as young individuals encouraged similar dietary behaviour throughout life (11-13). While no pre or post-menopausal human studies have been found that investigate the effects of goat milk on preventative bone loss, some work in children have shown that goats' milk increases skeletal mineralization and bone density (14-16).

Bone loss due to the onset of old age results from the uneven bone turnover. Formation of bone at the endosteal surface is halted and is coupled with the continued erosion of composite material from the endosteum. Over the years considerable research has gone into the development and use of anti-resorptive pharmacological treatments such as the bisphosphonate drug, sodium alendronate. This drug has been shown to be an effective tool in arresting bone resorption from the endosteal surface of osteoporotic bone in both animal and human trials (17, 18). It does this by binding to the hydroxyapatite crystals in the bone. This is primarily done at bone turnover sites preventing the resorption of the apatite crystals.

Dietary and medical treatments may be complementary in their effects on osteoporosis. Therefore treatments that arrest any undue increase in the marrow cavity size of the femur of the ovariectomized rat, may also allow greater mineralization from dietary supplements such as goats' milk to take place. No studies could be found that have investigated the relationship between goat milk as a nutritional supplement and sodium alendronate. Nor could any studies be found that compared the effects of goat and cow's milk supplemented diets in the ovariectomized rat model. The aim of this study

was to determine in the long term whether use of goat milk from weaning assists in offsetting the morphological and mineral changes known to accompany ovariectomy in the mature female rat. This study investigated whether the co-administration of goat milk diet and the bisphosphonate drug sodium alendronate further assisted in offsetting osteoporosis like conditions in the ovariectomized mature rat.

5.2. Methods & materials

5.2.1. Animals

One hundred and forty Sprague-Dawley female rats were individually caged and grown alongside the rats described in chapter 3. The rats were divided into three groups as weanlings (3 weeks of age) and fed the non-milk diet (60 rats), goat milk diet (40 rats) or cow's milk diet (40 rats). At 20 weeks of age the rats were introduced to this trial and were divided into a total of seven groups (Table 5-1).

Table 5-1. Seven animal groups in the trial

Diet Group	Non-milk			Cow		Goat	
	Sham	Ovx	Ovx ald	Cow ovx	Cow ovx ald	Goat ovx	Goat ovx ald
Rats/group	20	20	20	20	20	20	20

At 22 weeks of age rats were sham operated (SHAM) or ovariectomized (OVX). At 30 weeks of age the Alendronate (ALD) drug treatment was introduced to the diet Non-milk group (OVX ALD), the Goat milk diet group (GOAT OVX ALD), and the Cow's milk diet group (COW OVX ALD). The remaining 20 ovariectomized rats from each diet group were given a placebo, Non-milk diet (OVX), Goat milk diet (GOAT OVX), and Cow's milk diet (COW OVX). The sham operated rats were also given the placebo.

At 22 weeks of age the rats underwent ovariectomy (OVX) or sham-ovariectomy surgery (SHAM). The rats were anaesthetised for surgery by inhalation of 1.5 - 2% Isofluorane. A midline incision was made half way between the hump of the back and the base of the tail. The skin was pulled half way down one side of the body and an incision made through the peritoneal muscles. The ovaries were exteriorized and the fallopian tube, uterine horn, ovarian blood vessels and fat were severed with a single cut through the distal part of the horn, and the horn returned to the abdominal cavity. A similar procedure was used for sham surgery, except that the ovaries were not exteriorised or removed.

5.2.2. Diets

Rats were feed a semi-synthetic diet with vitamin and minerals content for growing rats, based on AIN93G (19). The diets were balanced to match the composition of the goat milk diet in calcium 0.69% and phosphorus 0.59%. The protein was set at 15%, and fat content at 8.2% (Table 5-2). The protein source was egg albumin for the non-milk diet, goat whole and skim milk for the goat diet and cow whole and skim milk for the cow diet. Dietary intake was restricted to 20g/day in order to prevent the rats from becoming obese (20).

Table 5-2. Diet powder composition (%) for non-milk and experimental diet formulations.

	Treatment		
	Non-milk	Goat	Cow
Egg Albumin	21.3		
Goat (whole + skim milk)[1]		47.4	
Cow (whole + skim milk)[2]			46.3
Starch	59	44	45
Soya oil	8		
Cellulose	5	5	5
Vitamin mix[3]	1	1	1
Mineral mix + trace salts [4]	3.5		
Trace salts		2	2
Calcium Hydrogen phosphate ($CaHO_4P$)	2.2	0.6	0.7

[1] Goat milk protein ratio 57:43 whole milk powder:skim milk powder.
[2] Cow milk protein ratio 67:33 whole milk powder: skim milk powder.
[3] (mg kg^{-1} diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, $_{D}$-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, $_{D}$-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg kg^{-1} diet) ergocalciferol 25.0, cyanocobalamin 50.0.
[4] (g kg^{-1} diet) calcium 0.69, chloride 7.79, magnesium 1.06, potassium 4.86, phosphate 0.59, sodium 1.97; (mg kg^{-1} diet) chromium 1.97, copper 10.7, iron 424.0, manganese 78.0, zinc 48.2; (μg kg^{-1} diet) colbalt 29.0, iodine 151.0, molybdenum 152.0, selenium 151.0.

5.2.3. Drug treatment

Sodium Alendronate (supplied by Merck, Whitehouse Station, USA) was administered weekly to groups OVX ALD, GOAT OVX ALD and COW OVX ALD at a dose rate of 7mg/kg bodyweight/week. The dose was calculated weekly for each individual rat. It was dissolved in milli-Q water, then mixed into liquid raspberry jelly to make a total

volume of 3ml, (method based on Kuhn-Sherlock & Schollum, 2001) (21). Alendronate is recommended to be taken on an empty stomach, therefore rats were fed in the late afternoon at 4pm when food intake is believed to be minimal and their stomachs are most likely to be empty. Rats are feed daily between 8am and 10am at which time they generally consume all of their diet. The SHAM, OVX, GOAT OVX, AND COW OVX groups received a plain raspberry jelly placebo.

5.2.4. Bone densitometry by Duel energy X-ray Absorptiometry (DEXA)

At 20, 30 and 38 weeks of age weeks *in vivo* DEXA were performed to assess whole body, lumbar spine and femur measurements. The measurements were bone mineral density (BMD), bone mineral content (BMC), bone area, whole body fat mass, lean mass, bone mass, and % of body fat (PFAT). Measurements were taken using a "Hologic Discovery A" bone densitometer (Bedford, MA, USA). On each day that scans were undertaken, a quality control (QC) scan was taken to ensure that its precision met the required DEXA manufacturer's coefficient of variation. The coefficient of variation (CV) for the QC data was 0.98 – 1.01%. Coefficient of variance for the femurs with repositioning between the scans was 1.20%, and without repositioning the scans the coefficient was 0.60%. These values ranged between 0.61% and 1.38% for the lumbar spine.

Each rat underwent three regional high-resolution scans of the spine and left and right femurs. Before scanning, the rats were anaesthetised with a drug mixture of Ketamine, Acepromazine (ACP), sterile water, and Xylazine at a ratio of (5:2:2:1). The dose was administered via intra-peritoneal injection at 0.05ml/100g of body weight. Rats were positioned supine with right angles between the spine and femur, and between femur and tibia.

5.2.5. Euthanasia and sample collections

Upon completion of the trial at 38 weeks of age the rats were euthanized by exsanguinations under anaesthesia. The anaesthetic was administered at a dose rate of 0.1ml/100g of bodyweight, using the same mixture and ratio as described in the above section (DEXA). Both hind legs of each rat were subsequently removed by simple dissection, and placed in phosphate buffered saline (PBS) at -20° Celsius pending further analysis.

5.2.6. Femurs

Left femurs were thawed and their weight and length determined after any residual adherent soft tissue had been removed. The femurs were prepared and sliced using the method described in Chapter 3. Subsequent measurements were taken from the twelve slices (slices 5-16) that constituted the tubular portion of the femoral shaft. These slices spanned 60 % of the overall length, and extended from the upper limit of the intercondylar ridge (slice 5) at the distal end of the femoral shaft, to the upper limit of the third trochanter (slice 16) at the proximal end of the femoral shaft.

Each of the bone cross sectional surfaces was scanned using the same method described in Chapter 2 (Fig. 5-1).

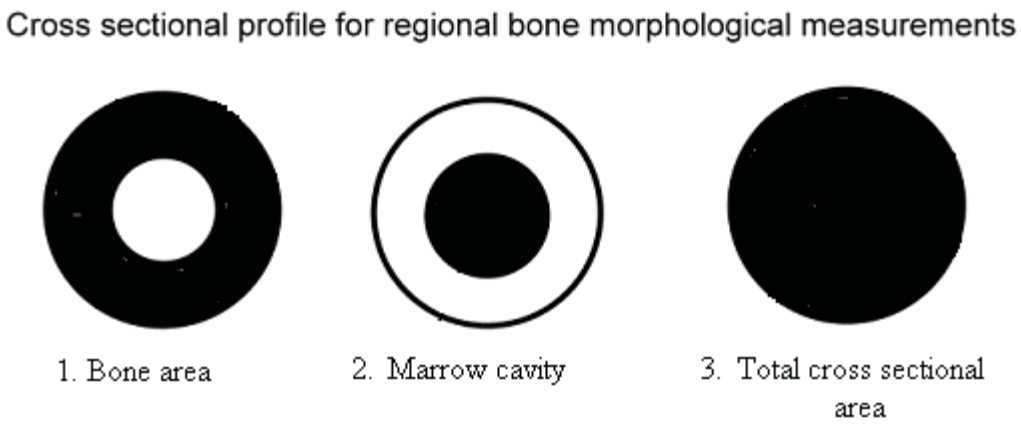

Figure 5-1. Picture describing the cross sectional profile of the femur slice.

1. Shows the area used to calculate the bone area (mm^2). 2. Shows the area used to calculate marrow cavity area (mm^2). 3. Shows the area used to calculate the total cross sectional area (mm^2).

5.2.7. Statistical analysis

Data were analysed in the statistical package "SAS" (version 9.3) (Sas Institute, Cary, USA). The mean bone area, mean marrow cavity area, and mean total cross sectional area of the slices taken from the femoral shaft were each normally distributed and were, therefore, amenable to parametric analysis. Uterus weights, bodyweight and diet intake data and DEXA were found for the most part to be normally distributed. Where non-

normal data were found a log transformation was required to obtain near normal distribution on graphic analysis.

Uterus weights and diet intake were examined by one-way ANOVA. Bodyweights and DEXA measurements were analyzed using General linear model (GLM) two-way ANOVA. The effects of diet and drug on bone area (mm^2), marrow cavity (mm^2), and total cross sectional area (mm^2) in the femoral shaft were compared by one way repeated measures ANOVA. The pattern of variation of each of these parameters between slices was explored using multivariate principal component analysis (PCA). A one way ANOVA was then conducted on the individual principal component scores in each axis of variation in order to statistically compare the effects of the diets. Data from incorrect scanning or damaged bone samples caused during preparation were removed from analysis. All tests used Tukey post-hoc testing and significance was set at ($p \leq 0.05$).

5.3. Results

Sixteen rats died during the trial due to a vitamin A deficiency (pg. 5.2) and other complications incurred during the trial. Diet compositions including calcium, phosphorus, percent fat and percent protein were confirmed by chemical analysis (data not shown).

Ovariectomy surgery was confirmed by extraction of the uterus post-mortem at 38 weeks of age. As expected, mean uterus weights were significantly higher in the sham rats compared to ovariectomized rats ($p<0.0005$). One outlier was shown in the ovariectomized rat population indicating a failed ovariectomy surgery. All data from that rat were removed from all analyses.

5.3.1. Duel energy X-ray Absorptiometry (DEXA)

Bodyweight and whole body composition

As a result of being raised on their selected diets from three weeks of age, the rats at the start of this trial already showed the significant differences similar to those reported in the previous chapter 4 (data not shown). At 20 weeks of age there were significant differences between the bodyweights of the rats fed the milk diets compared to the rats

fed the non-milk diet ($p<0.0005$). As expected, by the end of the trial at 38 weeks of age the SHAM rats fed the non-milk diet had the lowest bodyweights compared to OVX and OVX ALD rats fed the non-milk diet ($p<0.0001$). There was no difference in bodyweight between the groups fed either of the two milk diets; nor was there any interaction between milk diet or drug treatment.

Both OVX and OVX ALD rats fed the non-milk diet saw a significant rate of change throughout the trial in whole body area ($p<0.0001$), BMC ($p<0.0001$), fat ($p<0.0001$), bone mass ($p<0.0001$) and pfat ($p<0.0001$) (Table 5-3 a). The SHAM rats also had a significant rate of change over the trial period in whole body fat ($p<0.0303$), lean ($p<0.0465$), and bone mass ($p<0.0021$).

There was a significant interaction between treatment and week throughout the trial in whole body area ($p<0.0001$), BMC ($p<0.0001$), fat ($p<0.0039$) and pfat ($p<0.0014$) for the rats in the groups fed the non-milk diet. Post-hoc testing showed the whole body areas of the OVX rats and OVX ALD rats fed the non-milk diet, were significantly greater by the end of the trial compared to the SHAM rats ($p<0.0001$). Similarly the OVX rats also had significantly greater lean ($p<0.0282$) and bone mass ($p<0.0282$) than the SHAM rats fed the non-milk diet. The OVX ALD rats had greater whole body BMC than the OVX rats ($p<0.0001$) and the SHAM rats ($p<0.0004$) fed the non-milk diet.

Over the course of the trial there was significant change within each of the groups fed the milk diet in whole body area ($p<0.0001$), BMC ($p<0.0001$), and bone mass ($p<0.024$) (Table 5-3 b). The COW OVX ALD rats ($p<0.0023$), and GOAT OVX ALD rats ($p<0.0257$) also had a significantly greater rate of change in whole body BMD over the course of the trial. Similarly the COW OVX rats ($p<0.0001$) and GOAT OVX rats ($p<0.0001$) had a greater rate of change in whole body fat over the period of the trial.

Significant differences were found in the interaction between drug and week for femur BMD ($p<0.0036$). However, post-hoc testing did not reveal where these differences occurred.

Table 5-3. Repeated measures ANOVAs of Whole body area (cm^2), bone mineral content (BMC)(g), and bone mineral density (BMD) (g/cm^2), fat (g), lean (g), bone mass (g) and percentage fat (pfat).

a) The three rat groups fed the non-milk diet (SHAM, OVX and OVX ALD).

While body area (cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	12.92	**0.0001**
Week	1,45	101.20	**0.0001**
Week*Treatment	2,45	20.13	**0.0001**
While body BMC (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	9.45	**0.0004**
Week	1,45	53.16	**0.0001**
Week *Treatment	2,45	12.97	**0.0001**
While body BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	1.83	0.1727
Week	1,45	5.17	**0.0279**
Week *Treatment	2,45	0.23	0.7986
While body fat (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	1.53	0.2283
Week	1,45	96.86	**0.0001**
Week *Treatment	2,45	6.28	**0.0039**
While body lean (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	19.46	**0.0001**
Week	1,45	20.29	**0.0001**
Week *Treatment	2,45	0.18	0.8397
While body bone mass (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	9.73	**0.0003**
Week	1,45	62.39	**0.0001**
Week *Treatment	2,45	1.95	0.1539
While body pfat (%)			
Factor	df.	F- statistic	P- value
Treatment	2,45	0.58	0.5621
Week	1,45	99.80	**0.0001**
Week *Treatment	2,45	7.61	**0.0014**

b) The four rat groups fed the milk diets (GOAT OVX, GOAT OVX ALD, COW OVX, COW OVX ALD).

Whole body fat (g)

Factor	df.	F- statistic	P- value
Diet	1,75	0.47	0.4962
Drug	1,75	1.16	0.2859
Diet*Drug	1,75	2.07	0.1546
Week	1,75	92.60	**0.0001**
Diet*Week	1,75	6.02	**0.0165**
Drug*Week	1,75	0.80	0.3730
Diet*Drug*Week	1,75	0.00	0.9996

Whole body lean (g)

Factor	df.	F- statistic	P- value
Diet	1,75	3.29	0.0737
Drug	1,75	0.17	0.6827
Diet*Drug	1,75	3.93	0.0511
Week	1,75	9.61	**0.0027**
Diet*Week	1,75	0.40	0.5265
Drug*Week	1,75	3.79	0.0555
Diet*Drug*Week	1,75	0.54	0.4661

Whole body bone mass (g)

Factor	df.	F- statistic	P- value
Diet	1,75	0.14	0.7097
Drug	1,75	1.20	0.2768
Diet*Drug	1,75	0.79	0.3761
Week	1,75	80.37	**0.0001**
Diet*Week	1,75	3.16	0.0794
Drug*Week	1,75	0.07	0.7927
Diet*Drug*Week	1,75	0.07	0.7927

Whole body pfat (%)

Factor	df.	F- statistic	P- value
Diet	1,75	1.43	0.2356
Drug	1,75	1.28	0.2608
Diet*Drug	1,75	4.34	**0.0405**
Week	1,75	91.63	**0.0001**
Diet*Week	1,75	4.75	**0.0324**
Drug*Week	1,75	2.02	0.1592
Diet*Drug*Week	1,75	0.13	0.7181

Whole body area (cm^2)

Factor	df.	F- statistic	P- value
Diet	1,75	0.00	0.9546
Drug	1,75	0.76	0.3870
Diet*Drug	1,75	0.99	0.3238
Week	1,75	126.82	**0.0001**
Diet*Week	1,75	2.30	0.1335
Drug*Week	1,75	0.21	0.6516
Diet*Drug*Week	1,75	0.32	0.5708

Whole body BMC (g)

Factor	df.	F- statistic	P- value
Diet	1,75	0.03	0.8587
Drug	1,75	0.14	0.7119
Diet*Drug	1,75	0.54	0.4644
Week	1,75	184.19	**0.0001**
Diet*Week	1,75	0.95	0.3333
Drug*Week	1,75	0.37	0.5453
Diet*Drug*Week	1,75	0.19	0.6677

Whole body BMD (g/cm^2)

Factor	df.	F- statistic	P- value
Diet	1,75	0.31	0.5799
Drug	1,75	0.85	0.3588
Diet*Drug	1,75	0.28	0.6005
Week	1,75	27.47	**0.0001**
Diet*Week	1,75	1.01	0.3192
Drug*Week	1,75	4.56	**0.0360**
Diet*Drug*Week	1,75	0.06	0.8058

Lumbar spine

Over the course of the trial there was no significant change within each of the groups fed the non-milk diet in lumbar spine area, BMC, or BMD (Table 5-4). However by 38 weeks of age there was a significantly greater loss in BMD from the OVX ALD rats ($p<0.0045$) and OVX rats ($p<0.0007$) fed the non-milk diet compared to the SHAM rats fed the non-milk diet.

Significant differences were found in the interaction between drug and week for lumbar spine BMD ($p<0.0016$) and BMC ($p<0.0013$) (Table 5-4). However, post-hoc testing did not reveal where these differences occurred. There was an effect of time on lumbar spine BMD ($p<0.0001$). Further testing showed that the COW OVX rats ($p<0.0084$) and GOAT OVX rats ($p<0.0048$) lost BMD at a greater rate over time, whereas there was no significant rate of change in the rats dosed with alendronate for either of the milk groups.

Table 5-4. Repeated measures ANOVAs of Lumbar spine bone area (cm^2), bone mineral content (BMC) (g), and bone mineral density (BMD) (g/cm^2).

a) The three rat groups fed the non-milk diet (SHAM, OVX and OVX ALD).

Lumbar spine area (cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	0.83	0.4428
Week	1,45	10.43	**0.0023**
Week*Treatment	2,45	2.76	0.0739
Lumbar spine BMC (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	6.28	**0.0039**
Week	1,45	2.14	0.1505
Week *Treatment	2,45	0.66	0.5205
Lumbar spine BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	16.24	**0.0001**
Week	1,45	1.01	0.3208
Week *Treatment	2,45	0.65	0.5260

b) The four rat groups fed the milk diets (GOAT OVX, GOAT OVX ALD, COW OVX, COW OVX ALD).

Lumbar spine area (cm^2)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.26	0.6090
Drug	1,75	0.25	0.6216
Diet*Drug	1,75	0.36	0.5528
Week	1,75	3.81	0.0547
Diet*Week	1,75	0.03	0.8602
Drug*Week	1,75	3.48	0.0662
Diet*Drug*Week	1,75	0.03	0.8709
Lumbar spine BMC (g)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.14	0.7094
Drug	1,75	0.49	0.4870
Diet*Drug	1,75	0.59	0.4456
Week	1,75	3.17	0.0790
Diet*Week	1,75	0.28	0.5974
Drug*Week	1,75	11.10	**0.0013**
Diet*Drug*Week	1,75	0.10	0.7526
Lumbar spine BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.00	0.9843
Drug	1,75	2.07	0.1541
Diet*Drug	1,75	0.52	0.4742
Week	1,75	19.76	**0.0001**
Diet*Week	1,75	0.67	0.4141
Drug*Week	1,75	10.70	**0.0016**
Diet*Drug*Week	1,75	0.25	0.6195

SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19).

Femur

There was a significant interaction between time and surgery on femur BMD, with both OVX ($p<0.0001$) and OVX ALD ($p<0.0102$) rats fed the non-milk diet having a greater rate of loss than the SHAM rats fed the non-milk diet over the course of the trial (Table 5-5 a). This was also true for femur area, where the SHAM rats had smaller changes in area than either the OVX ($p<0.0305$) or OVX ALD ($p<0.03789$) rats fed the non-milk diet.

Significant differences were found in the interaction between drug and week for femur BMD ($p<0.0028$) for the rats fed the milk diets (Table 5-5 b). However, post-hoc testing did not reveal where these differences occurred.

Table 5-5. Repeated measures ANOVAs of Femur bone area (cm^2), bone mineral content (BMC)(g), and bone mineral density (BMD) (g/cm^2).

a) The three rat groups fed the non-milk diet (SHAM, OVX and OVX ALD).

Femur area (cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	10.44	**0.0002**
Week	1,45	5.51	**0.0233**
Week*Treatment	2,45	1.33	0.2803
Femur BMC (g)			
Factor	df.	F- statistic	P- value
Treatment	2,45	1.28	0.2881
Week	1,45	0.04	0.8393
Week *Treatment	2,45	4.54	**0.0160**
Femur BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Treatment	2,45	21.39	**0.0001**
Week	1,45	7.55	**0.0086**
Week *Treatment	2,45	4.64	**0.0147**

b) The four rat groups fed the milk diets (GOAT OVX, GOAT OVX ALD, COW OVX, COW OVX ALD).

Femur area (cm^2)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.87	0.3539
Drug	1,75	0.96	0.3309
Diet*Drug	1,75	0.35	0.5552
Week	1,75	1.23	0.2702
Diet*Week	1,75	0.00	0.9941
Drug*Week	1,75	0.45	0.5023
Diet*Drug*Week	1,75	1.21	0.2742
Femur BMC (g)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.37	0.5430
Drug	1,75	0.82	0.3688
Diet*Drug	1,75	0.44	0.5077
Week	1,75	0.47	0.4952
Diet*Week	1,75	1.08	0.3019
Drug*Week	1,75	2.19	0.1429
Diet*Drug*Week	1,75	1.86	0.1764
Femur BMD (g/cm^2)			
Factor	df.	F- statistic	P- value
Diet	1,75	0.11	0.7448
Drug	1,75	4.81	**0.0314**
Diet*Drug	1,75	0.81	0.3723
Week	1,75	0.34	0.5633
Diet*Week	1,75	2.12	0.1493
Drug*Week	1,75	9.52	**0.0028**
Diet*Drug*Week	1,75	0.16	0.6883

SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19).

5.3.2. Morphology of the femoral shaft

Bone area (mm^2)

The overall bone area of the slices from the femoral shaft differed significantly on repeated measures ANOVA between treatment groups ($p< 0.0005$) (Table 5-6). There were significant differences on ANOVA between slices in bone area ($p< 0.0005$), and the treatments did have a significant effect between slices ($p<0.0005$). This indicated there were significant differences between treatments in bone area of the different regions in the femoral shaft.

Table 5-6. Repeated measures ANOVAs of overall bone area (mm^2), marrow cavity area (mm^2), and total cross sectional area (mm^2) of slices of the femoral shaft from the seven groups.

Bone area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	6,82	9.612	**0.0005**
Within subjects			
Slice	11,902	747.490	**0.0005**
Slice*Treatment	66,902	2.439	**0.0005**
Marrow cavity area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	6,94	11.190	**0.0005**
Within subjects			
Slice	11,1034	823.347	**0.0005**
Slice*Treatment	66,1034	6.836	**0.0005**
Total cross sectional area (mm^2)			
Between subjects	df.	F- statistic	P- value
Treatment	6,90	11.835	**0.0005**
Within subjects			
Slice	11,990	484.755	**0.0005**
Slice*Treatment	66,990	6.764	**0.0005**

SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19). Non-milk represents the rats fed the non-milk diet, Cow represents the rats fed the cow's milk based diet, and Goat represents the rats fed the goat milk based diet.

Table 5-7. Table of bone area (mm^2) and component loadings from a principal component analysis of the 12 slices of the femoral shaft.

Slice	Bone area (mm^2) Component loadings	
	PC1	PC2
16	0.771	-0.385
15	0.805	-0.438
14	0.849	-0.376
13	0.891	-0.286
12	0.928	-0.182
11	0.942	-0.119
10	0.947	0.022
9	0.935	0.157
8	0.911	0.260
7	0.861	0.392
6	0.813	0.518
5	0.725	0.469
Percentage of total variation	**75.261**	**11.207**

Component loadings from principal component analysis for the 12 slices of the femoral shaft for the seven treatment groups fed three different diets. PCA showed two patterns of variation, PC1 accounted for 75.3% of that variance and PC2 accounted for 11.2%. All component weightings of PC1 had large positive values, indicating variation in overall size. PC2 reflected a contrast between bone areas in the proximal and distal ends of the shaft i.e. a difference in the pattern of distribution of bone area along the femoral shaft. The magnitudes of the component loadings were greater in the slices from the two ends and lower in the more centrally situated slices. SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19). *Means and SD's for the slices are given in Table 5-10 (pg. 5-32).*

Principal component analysis (PCA) showed two patterns of variation for bone area in the 12 slices of the femoral shaft (Table 5-7). Combined these two components explained 86% of the total variation in the data. The effect of treatment on each of the PC scores was investigated by one-way ANOVA. PC1 scores showed significant differences between treatments ($p<0.0005$). The overall bone area of the GOAT OVX milk group was significantly larger than all groups, except OVX ALD and COW OVX ALD. While the difference was not significant between GOAT OVX and GOAT OVX ALD, there was a tendency for GOAT OVX to have a larger overall bone area (p<

0.063). Furthermore, the SHAM operated group was sufficiently smaller in overall bone area than all groups except OVX rats. One way ANOVA of PC2 scores gave significant differences between treatment groups ($p< 0.052$), however, post hoc testing did not show these differences. Further investigation, by principal component analysis, of the proximal and distal regions of the femoral shaft showed that SHAM rats had significantly smaller bone area in the distal region to all groups ($p<0.05$) except for GOAT OVX ALD (data not shown). The proximal region also showed sham with a significantly smaller bone area to the milk groups, and GOAT OVX and COW OVX ALD having the largest. COW OVX ALD ($p<0.033$) and GOAT OVX ($p<0.017$) also had a significantly larger bone area than COW OVX.

Marrow cavity area (mm^2)

The overall marrow cavity areas of the slices from the femoral shaft differed significantly on repeated measures ANOVA between treatment groups ($p< 0.0005$) (Table 5-6). There were significant differences on ANOVA between slices in marrow cavity area ($p< 0.0005$) and the treatments did have a significant effect between slices ($p<0.0005$). This indicated that the treatments impacted on the regional pattern of marrow cavity area size throughout the shaft of the femur.

Table 5-8. Table of for marrow cavity area (mm^2) and component loadings from a principal component analysis of the 12 slices of the femoral shaft.

Slice	Marrow cavity area (mm^2) Component loadings	
	PC1	PC2
16	0.726	-0.535
15	0.801	-0.506
14	0.824	-0.506
13	0.877	-0.394
12	0.916	-0.283
11	0.932	-0.029
10	0.905	0.173
9	0.885	0.302
8	0.913	0.358
7	0.884	0.401
6	0.829	0.442
5	0.755	0.491
Percentage of total variation	**73.306**	**15.681**

Component loadings from principal component analysis for the 12 slices of the femoral shaft for the seven treatment groups fed three different diets. PC1 of a principal component analysis of marrow cavity area accounted for 73.3% of the variation in the data and reflected varying overall size. The second principal component (PC2) reflected 15.7 % of the variation in cavity shape according to location. The magnitude of the component loadings was greatest at the two ends and lower in the more centrally situated slices. SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19). *Means and SD's for the slices are given in Table 5-11 (pg. 5-33).*

PCA showed two patterns of variation for bone area in the 12 slices of the femoral shaft (Table 5-8). One way ANOVA of PC1 scores showed significant differences between treatments ($p < 0.0005$); the overall marrow cavity being larger in the OVX and OVX ALD rats compared to all other groups. PC2 factor scores showed increased growth within the proximal region of OVX rats compared to SHAM ($p<0.0005$) and OVX ALD ($p<0.045$). Compared to the rats fed the milk diets the SHAM rats had significantly greater growth in the distal region ($p<0.001$) except the GOAT OVX ALD rats who only had a tendency to have a smaller distal marrow cavity ($p<0.064$).

Total cross sectional area (mm^2)

The overall cross sectional area of the slices from the femoral shaft differed significantly on repeated measures ANOVA between treatment groups ($p< 0.0005$) (Table 5-6). There were significant differences on ANOVA between slices in cross sectional area ($p< 0.0005$) and the treatments did have a significant effect between slices ($p<0.0005$). This indicated that the treatments impacted on the regional pattern of total size throughout the shaft of the femur.

Table 5-9. Table of for total cross sectional area (mm^2) and component loadings from a principal component analysis of the 12 slices of the femoral shaft.

Slice	Total Cross sectional area (mm^2) Component loadings	
	PC1	PC2
16	0.854	-0.391
15	0.877	-0.396
14	0.895	-0.400
13	0.909	-0.370
12	0.950	-0.259
11	0.965	-0.143
10	0.970	0.012
9	0.950	0.190
8	0.928	0.297
7	0.876	0.450
6	0.803	0.569
5	0.705	0.625
Percentage of total variation	**79.786**	**14.468**

Component loadings from principal component analysis for the 12 slices of the femoral shaft for the seven treatment groups fed three different diets. PC1 of a principal component analysis of total femoral cross sectional area accounted for 79.8% of the variation in overall size of the femurs. PC2 accounted for 14.5 % of that variance, and reflected a lengthwise variation in overall shape. The magnitudes of the component loadings and cross sectional area were greater in the slices from the two ends, and lower in the more centrally situated slices. SHAM = sham operated rats (n = 15), OVX = non-milk ovariectomized rats (n = 14), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 9). Non-milk represents the rats fed the non-milk diet, *Means and SD's for the slices are given in Table 5-12 (pg. 5-34).*

PCA showed two patterns of variation for cross sectional area in the 12 slices of the femoral shaft (Table 5-9). Combined these two components explained 94% of the total variation in the data. One way ANOVA of PC1 scores showed significant differences between treatments within the overall size of the femoral shaft ($p<0.0005$). OVX and OVX ALD rats had significantly larger sized femurs overall than all other groups. A one way ANOVA of PC2 scores also showed significant differences between treatments ($p<0.0005$). PC2 showed that there was significantly more variation between the proximal and distal regions of the femoral shaft within the SHAM operated, OVX and OVX ALD groups compared to all the groups fed the milk diets. Further investigation of proximal and distal regions showed that same pattern in the distal region. However in the proximal region there was no significant difference between OVX and OVX ALD with the milk groups (data not shown). The proximal region also showed that there was no drug effect in any of the groups. This suggests that the milk diet groups showed a more uniform shape between the different regions of the femoral shaft.

5.4. Discussion

This study investigated the complementary effects of the long term consumption of a goat milk based diet and a cow's milk based diet with and without Alendronate in ovariectomized rats. As a pre-condition for this trial the rats used were grown from weanlings (3 weeks of age) on either a goat milk based diet, cow's milk based diet or a non-milk diet. As the rats fed the milk diets entered this trial at 20 weeks of age they showed improved body condition and bone health compared to the rats fed the non-milk diet. However, by the conclusion of this trial it was discovered that the rats fed the non-milk diet had suffered from a vitamin A deficiency in their diet. The diet deficiency impacted on the study resulting in deaths due to what can only be called a 'serendipitous cascade of events', including a failed heating system at the animal housing facility. Infection and death resulted from a depressed immune system associated to the diet deficiency, cold temperatures, and was exacerbated by ovariectomy surgery.

Traditionally SHAM rats have been reported as having lower bodyweights compared to ovariectomized rats on the same diet. The weights of the rats fed the formulated goat milk diet in Kruger *et al* (2008) were similar to the sham-operated rats of that trial (22), whereas in this trial the rats fed the goat milk diet where comparatively heavier. Reasons for this difference can only be speculation as Kruger *et al* (2008) did not conduct whole body compositions with their DEXA analysis, and there are no other ovariectomized rats trials investigating the effects of goat milk. However, in the case of the current study, differences in body weight between the SHAM rats and the rats fed the goat milk diet are possibly due to the reduced weight gain known to accompany animals that have a vitamin A deficiency (23). The reason the other diet groups were less affected was due to the milk diets already containing vitamin A.

5.4.1. Diet deficiency impact on the femur

Ovariectomy surgery is known to change the morphological features of the femur by a larger marrow cavity from increased endosteal resorption, and larger overall femurs due to increased periosteal apposition. The changes in the size and shape of the femoral shaft found in the current study between the SHAM operated rats and OVX rats, follow the same pattern of effect to those found in 'Chapter 3'. However, it should be mentioned that area measurements from the current study show that both the SHAM

and OVX rat femurs were smaller in overall size and bone area, compared to the same groups from the previous trial. Also, in the current trial the rats fed the milk diets had a more uniform effect of periosteal apposition and endosteal resorption within the femoral shaft, whereas the rats on the non-milk diet tended to lose bone at an uneven rate throughout the shaft. These differences could be the result of the dietary deficiency found in the non-milk diet groups. Previous reports, however, indicate that the effects of vitamin A deficiency in the long bones of rats occurs in the epiphyses region (24), where continued formation at the periosteal surface and cessation of growth in the epiphyses result in shorter thicker long bones (24, 25).

Comparing the DEXA results of SHAM and CON OVX rats from the current trial with that of previous research shows that the femur BMC and BMD values of this trial fall into the lower ranges given by Kruger *et al* (2005), Poulsen *et al* (2007), and Kruger *et al* (2008). They reported the femur BMC for SHAM operated rats ranged from 0.48 to 0.53 (g), and 0.43 to 0.48 (g) for the control OVX rats. They also reported higher femur BMD values for SHAM rats ranging from 0.30 to 0.33 (g/cm^2), and 0.27 to 0.30 (g/cm^2) for the control OVX rats (22, 26, 27). The lower bone mineral densities found in the femurs of the current trial may be a reflection of the decreased femur size due to the vitamin A deficiency discussed above. The above studies used the same breed of rat, similar age at ovariectomy, euthanasia, and time of bone scan.

5.4.2. Diet and Alendronate effect on the ovariectomized rat

No studies could be found that compared the effects of a goat milk diet versus a cow's milk diet in ovariectomized rats, and only one study could be found that investigated goat milk. That study used a similar long term diet protocol as the current trial, and raised the rats on their diets from weanlings before entering the ovariectomized phase of the trial at 6 months of age. The investigators of that trial found that the rats fed the formulated goat milk diet had increased peak bone mass compared to the rats fed a soy-based diet, and went on to maintain a greater bone mass after ovariectomy (22). The current trial seems to follow this trend except that the SHAM rats would have been expected to follow a similar trend as was seen in the ovariectomy trial conducted in Chapter 3. The SHAM rats in the Kruger *et al* (2008 trial) had significantly greater BMC, BMD, femur mineral composition, larger femoral shaft bone areas compared to all of the ovariectomized rat groups (22). There have been several case studies done

with humans looking at the effect of milk consumption early in life and comparing that with adult BMD and fracture rates. In the USA a survey in the form or a questionnaire was taken of white women aged 20 years and over to determine milk intake over the participants' life time. The study included DEXA scans of the participants left hip and from the results they found that women with low milk intake during childhood and adolescence had less bone mass in adulthood (9). They suggested that the low intake of milk increased the risk of osteoporotic fracture (9). In contrast to this Feskanich *et al* (1997) suggested that long term milk consumption was not associated with the incidence of bone fracture in adults (28). Although, they did concede that while not significant, there was a possible relationship between milk consumption during adolescence and reduced fracture risk in adults (28).

DEXA measurements of the lumbar spine and femur did not show any significant differences between ovariectomized and ovariectomized + alendronate groups fed either of the milk diets. However, there was a potentially differing, almost opposite effect within each of the two milk diets in the bone area of the femoral shaft. The GOAT OVX rats had larger overall mean bone areas than the GOAT OVX ALD rats ($p<0.063$), yet in contrast to this the COW OVX rats had smaller overall mean bone areas than the COW OVX ALD rats in the femoral shaft, although not significant. This suggested that goat milk alone was sufficient to significantly increase the overall bone area of the femoral shaft in rats, while the COW OVX rats needed to be dosed with alendronate to achieve a similar result. Unfortunately current literature on the effects of alendronate on bone formation does not shed any light on the effects found here. It has been earlier suggested that Bisphosphonates inhibit bone formation in *in vitro* osteoblast cells and suppressed periosteal expansion in the mid-shaft of femurs in young male castrated rats (29, 30). Therefore, the question remains, did dosing the GOAT OVX rats with alendronate suppress periosteal bone formation resulting in smaller bone areas? If that was the case why did it not have the same effect on the rats fed the cow's milk diet and dosed with alendronate?

Closer examination of the marrow cavity area showed all four groups in the two milk diets had similar overall cavity sizes. This lack of any significant differences between the ovariectomized and ovariectomized + alendronate groups suggested that alendronate did not alter the morphology of the marrow cavity. Current theories indicate that

alendronate does not inhibit bone formation at the endosteal surface of long bones, and has been found to reduce the incidence of resorption at this site (30). It may not be advisable to compare the marrow cavities of the rats from the milk diets with the SHAM rats of this trial. However, comparing the overall mean marrow cavity areas of SHAM rats from 'Chapter 3' (47.3 ± 3.62) and that of the ovariectomized rats fed the milk diets in the current trial indicated that they had similar sized cavities. This suggests that the rats fed the milk diets were less affected by ovariectomy surgery than either the OVX rats from 'Chapter 3' or the OVX or OVX ALD rats of the current trial. This may be a reflection of what Kruger *et al* (2008) saw when they reported that the rats raised on the formulated goat milk diet maintained higher bone mass than the ovariectomized rats fed the soya-based diet (22). The regional variations in marrow cavity size between the proximal and distal ends of the femoral shaft were more pronounced in the ovariectomized + alendronate groups, although this was only significant in the non-milk diet group (OVX ALD). According to Lin *et al* (1991), the bisphosphonate drug ALD has an uneven distribution in bone; they found significantly higher proportions of the drug in the joints of the tibia and femur compared to the mid sections of the same bones (31). Further investigation of the levels of alendronate in the different regions of the shaft would have to be done to confirm whether or not this was the case here. Therefore, changes in bone area were not noticeably occurring at either the endosteal or periosteal surface. What would be of interest would be to determine if the morphological changes seen had any effect on the biomechanical competence of the bone, i.e. would these variations result in tougher or weaker bone?

5.4.3. Conclusion

DEXA measurements of the lumbar spine and femur did not show any significant differences between OVX and OVX ALD groups fed either of the milk diets suggesting that both goat and cow's milk diets with and without alendronate have similar effects on osteoporotic bone.

The GOAT OVX ALD rats had smaller overall mean bone areas than the GOAT OVX rats ($p<0.063$) possibly due to bone formation suppression at the periosteal surface by alendronate. There was a potentially differing, almost opposite effect within each of the two milk diets in bone area measurements of the femoral shaft. The mechanism to explain this apparent differing effect on the bone area remains unclear.

It is difficult to draw any conclusions from this work as the effects of the vitamin A deficiency meant it was no longer possible to compare the effects of the milk diets with a control diet. Therefore it is difficult to say whether either of the milk diets can into this trial with improved peak bone mass due to a diet effect or as a result of the deficiency. This problem is carried throughout this study post ovariectomy.

5.5. References

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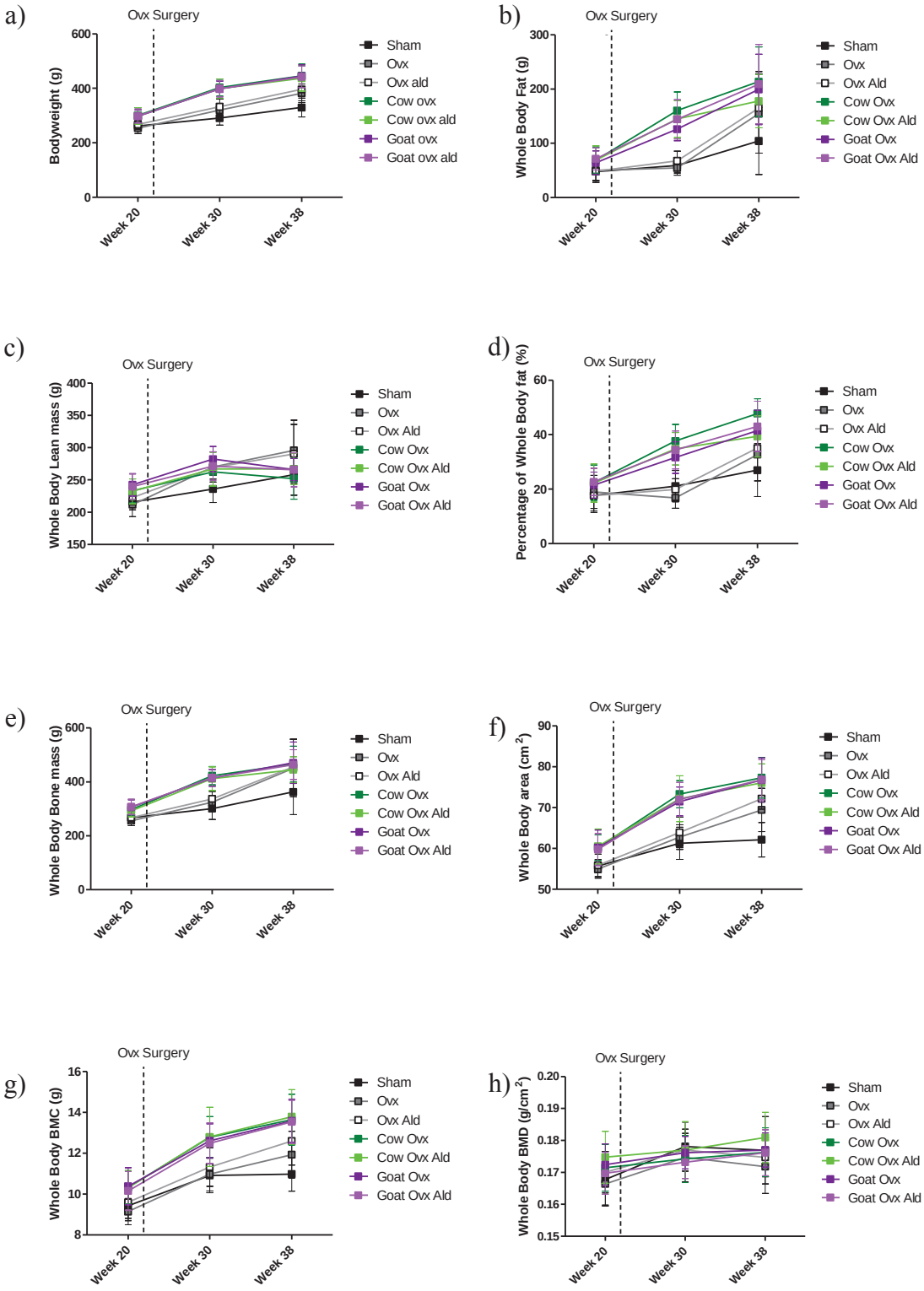


Figure 5-2. Graphs showing means and SD for Bodyweights and the seven DEXA whole body composition measurements taken at 20, 30, and 38 weeks of age for the seven treatment groups.

SHAM = sham operated rats (n = 17), OVX = non-milk ovariectomized rats (n = 13), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 18), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 20), COW OVX ALD = cow ovariectomized + alendronate rats (n = 20).

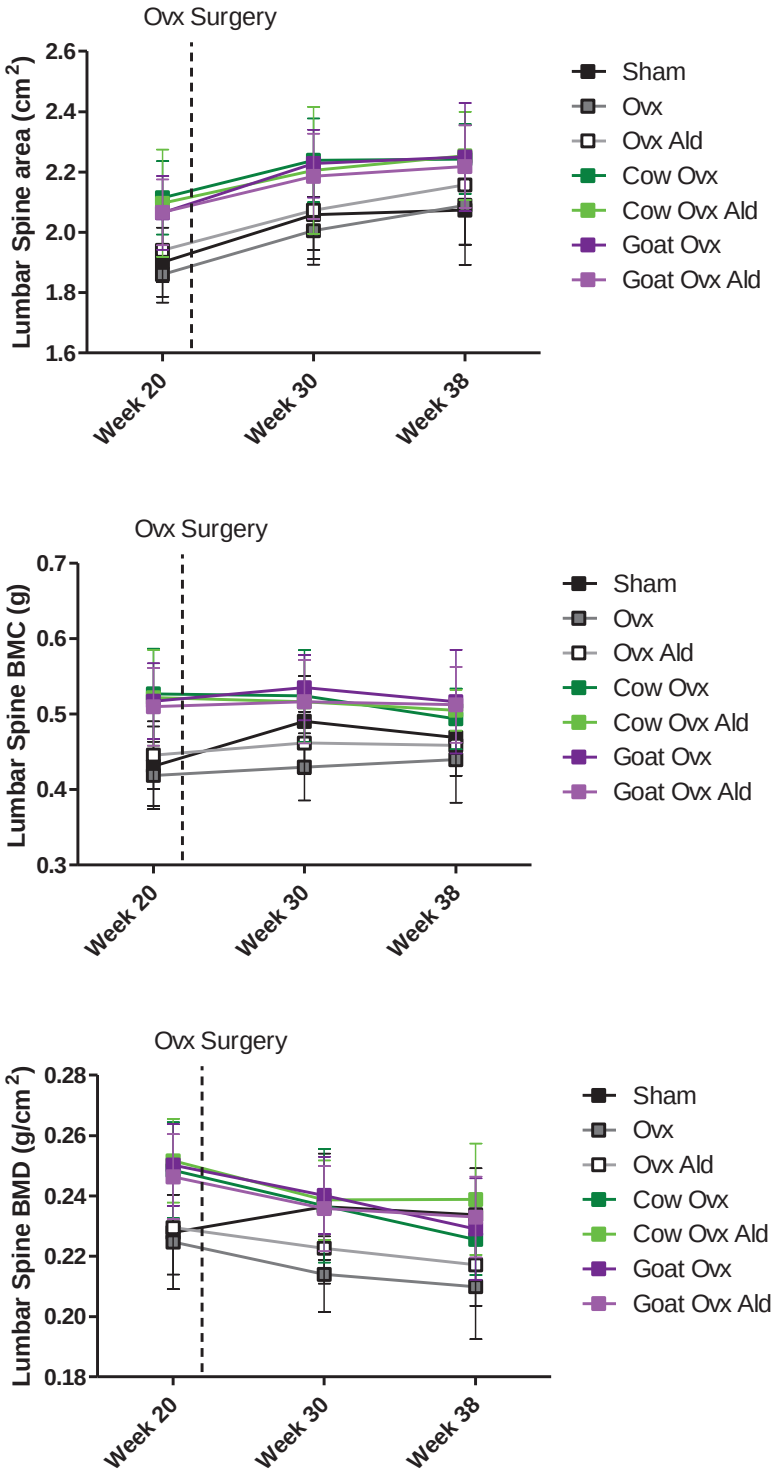


Figure 5-3. Graphs showing Lumbar Spine BMC, and BMD at 20, 30, and 38 weeks of age for the seven treatment groups.

SHAM = sham operated rats (n = 16), OVX = non-milk ovariectomized rats (n = 13), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 19), COW OVX ALD = cow ovariectomized + alendronate rats (n = 19).

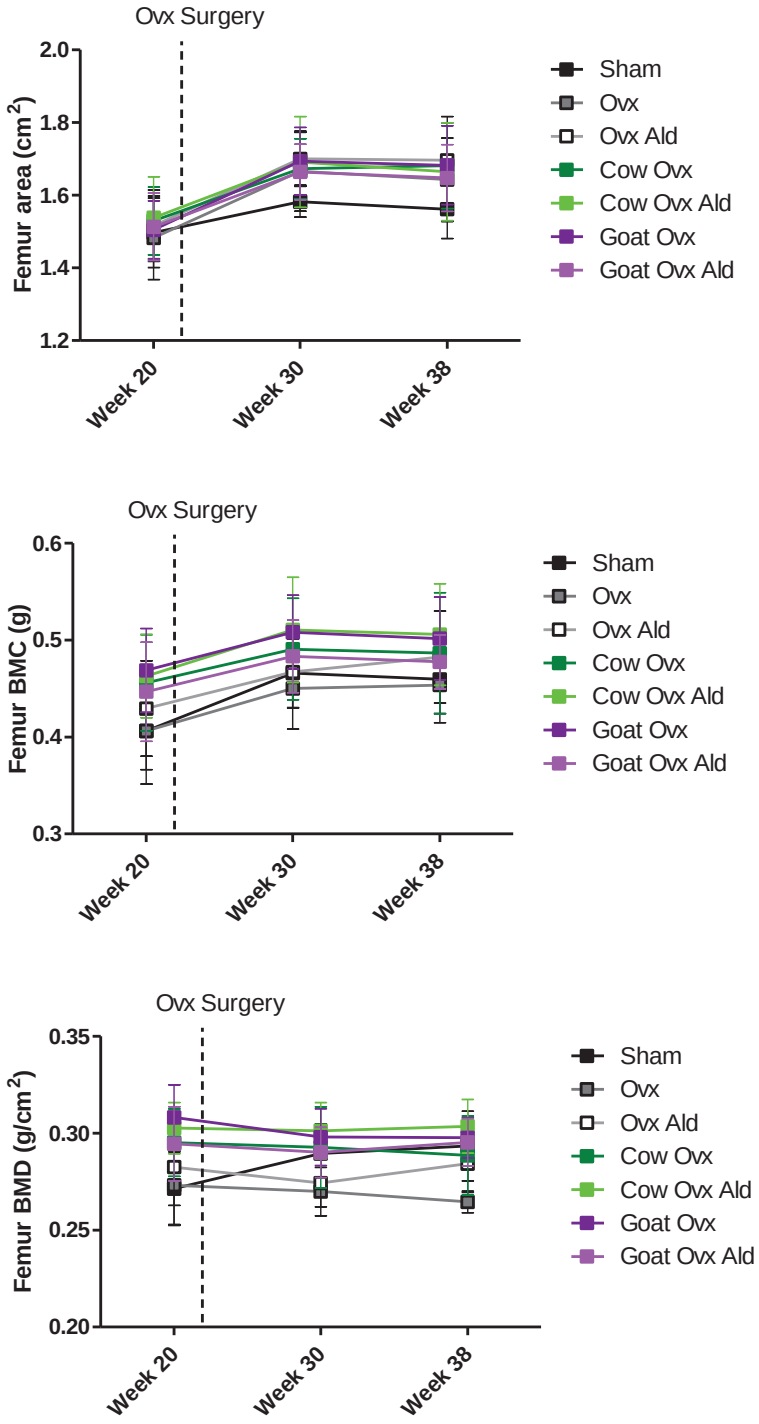


Figure 5-4. Graphs showing Femur BMC, and BMD at 20, 30, and 38 weeks of age for the seven treatment groups.

SHAM = sham operated rats (n = 16), OVX = non-milk ovariectomized rats (n = 12), OVX ALD = non-milk ovariectomized + alendronate rats (n = 14), GOAT OVX = goat ovariectomized rats (n = 19), GOAT OVX ALD = goat ovariectomized + alendronate rats (n = 19), COW OVX = cow ovariectomized rats (n = 20), COW OVX ALD = cow ovariectomized + alendronate rats (n = 20).

Table 5-10. Means and Standard deviations (SD's) for the Bone areas of the 16 slices of the femoral shaft.

	NON-MILK						GOAT				COW			
	N	Sham	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD
Slice 16	15	8.9 ± 0.73	14	8.8 ± 0.90	13	9.5 ± 0.88	20	9.2 ± 1.12	18	9.9 ± 0.80	19	9.7 ± 0.79	18	9.5 ± 0.80
Slice 15	16	8.4 ± 0.78	14	8.6 ± 0.89	10	9.1 ± 0.26	19	8.7 ± 0.91	20	9.5 ± 0.77	18	9.5 ± 0.70	19	9.1 ± 0.77
Slice 14	14	7.8 ± 0.37	13	8.3 ± 0.91	14	8.7 ± 0.64	20	8.5 ± 0.79	18	8.8 ± 0.51	19	9.0 ± 0.64	17	8.5 ± 0.51
Slice 13	14	7.2 ± 0.34	14	7.7 ± 0.78	14	8.0 ± 0.49	20	8.0 ± 0.87	20	8.5 ± 0.75	20	8.2 ± 0.64	19	8.0 ± 0.75
Slice 12	14	6.9 ± 0.21	10	7.2 ± 0.31	14	7.7 ± 0.52	20	7.4 ± 0.73	20	7.9 ± 0.70	20	7.7 ± 0.64	19	7.5 ± 0.70
Slice 11	16	6.7 ± 0.42	13	7.2 ± 0.58	14	7.4 ± 0.44	20	7.2 ± 0.79	20	7.69 ± 0.63	20	7.4 ± 0.51	19	7.3 ± 0.63
Slice 10	13	6.4 ± 0.19	13	6.9 ± 0.56	14	7.2 ± 0.50	20	6.9 ± 0.70	20	7.3 ± 0.62	19	7.2 ± 0.45	19	6.9 ± 0.62
Slice 9	13	6.3 ± 0.16	11	6.8 ± 0.38	12	6.9 ± 0.30	20	6.7 ± 0.59	20	7.1 ± 0.56	20	6.9 ± 0.49	17	6.8 ± 0.56
Slice 8	16	6.3 ± 0.44	12	6.7 ± 0.30	14	7.0 ± 0.43	20	6.6 ± 0.48	20	7.0 ± 0.56	20	6.8 ± 0.46	19	6.6 ± 0.56
Slice 7	16	6.3 ± 0.41	14	6.7 ± 0.55	14	6.9 ± 0.38	20	6.4 ± 0.51	20	6.7 ± 0.49	20	6.6 ± 0.53	14	6.3 ± 0.49
Slice 6	14	6.0 ± 0.26	14	6.5 ± 0.56	14	6.7 ± 0.56	20	6.4 ± 0.48	20	6.6 ± 0.47	20	6.5 ± 0.60	17	6.3 ± 0.47
Slice 5	15	5.9 ± 0.38	13	6.4 ± 0.60	14	6.8 ± 0.52	20	6.6 ± 0.48	20	6.8 ± 0.54	20	6.7 ± 0.64	19	6.5 ± 0.54

SHAM = sham operated rats, OVX = non-milk ovariectomized rats, OVX ALD = non-milk ovariectomized + alendronate rats, GOAT OVX = goat ovariectomized rats, GOAT OVX ALD = goat ovariectomized + alendronate rats, COW OVX = cow ovariectomized rats, COW OVX ALD = cow ovariectomized + alendronate rats. Non-milk represents the rats fed the non-milk diet, Cow represents the rats fed the cow's milk based diet, and Goat represents the rats fed the goat milk based diet.

Table 5-11. Means and Standard deviations (SD's) for the Marrow cavity areas of the 16 slices of the femoral shaft.

	NON-MILK						GOAT				COW			
	N	Sham	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD
Slice 16	13	3.497±0.51	14	4.813±0.93	9	4.123±0.30	19	3.630±0.63	18	3.513±0.54	20	3.883±0.74	18	3.679±0.63
Slice 15	16	3.668±0.66	14	4.749±0.72	12	4.326±0.37	19	3.738±0.55	18	3.479±0.42	20	3.916±0.78	20	3.623±0.80
Slice 14	13	3.517±0.34	13	4.839±0.62	14	4.512±0.52	20	3.867±0.49	19	3.678±0.48	19	3.948±0.60	20	3.749±0.73
Slice 13	13	3.698±0.34	14	4.935±0.70	14	4.694±0.45	20	4.081±0.51	19	3.839±0.44	19	4.031±0.55	20	3.890±0.74
Slice 12	16	4.043±0.71	14	4.852±0.74	14	4.699±0.43	20	4.095±0.42	19	3.847±0.55	19	4.072±0.55	19	3.855±0.49
Slice 11	16	4.091±0.70	13	4.755±0.78	14	4.692±0.52	20	3.982±0.44	19	3.723±0.60	20	3.961±0.58	19	3.718±0.47
Slice 10	16	4.188±0.71	14	4.663±0.84	14	4.693±0.59	20	3.959±0.46	19	3.751±0.62	20	3.850±0.57	19	3.651±0.51
Slice 9	16	4.320±0.80	13	4.491±0.57	14	4.818±0.63	20	3.896±0.42	19	3.766±0.69	19	3.779±0.44	19	3.657±0.56
Slice 8	16	4.654±0.91	14	5.175±0.91	14	5.174±0.80	20	3.964±0.43	19	3.829±0.71	20	3.920±0.67	19	3.755±0.56
Slice 7	16	5.427±1.09	14	6.133±1.06	14	6.207±1.06	20	4.476±0.48	19	4.472±0.82	20	4.392±0.67	20	4.400±0.97
Slice 6	16	7.172±1.50	14	7.788±1.41	14	7.813±1.34	18	5.695±0.50	18	5.709±0.98	20	5.565±0.90	20	5.733±1.33
Slice 5	16	10.155±1.84	14	10.399±2.29	14	10.394±1.67	20	7.903±0.91	19	8.081±1.20	20	7.768±1.25	20	8.180±1.93

SHAM = sham operated rats, OVX = non-milk ovariectomized rats, OVX ALD = non-milk ovariectomized + alendronate rats, GOAT OVX = goat ovariectomized rats, GOAT OVX ALD = goat ovariectomized + alendronate rats, COW OVX = cow ovariectomized rats, COW OVX ALD = cow ovariectomized + alendronate rats. Non-milk represents the rats fed the non-milk diet, Cow represents the rats fed the cow's milk based diet, and Goat represents the rats fed the goat milk based diet.

Table 5-12. Means and Standard deviations (SD's) for the Cross sectional areas of the 16 slices of the femoral shaft.

Diet	NON-MILK						GOAT				COW			
	N	Sham	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD	N	OVX	N	OVX ALD
Slice 16	12	12.1 ±0.112	14	13.6 ±0.415	13	13.7 ±0.394	19	13.3 ±0.249	18	13.0 ±0.219	20	13.1 ±0.316	17	13.4 ±0.261
Slice 15	13	11.6 ±0.145	14	13.4 ±0.377	14	13.7 ±0.297	19	13.1 ±0.225	19	12.7 ±0.200	19	12.6 ±0.259	19	13.0 ±0.245
Slice 14	11	11.3 ±0.086	13	13.1 ±0.388	14	13.2 ±0.254	20	12.7 ±0.222	19	12.3 ±0.190	19	12.4 ±0.215	16	12.8 ±0.181
Slice 13	12	11.0 ±0.100	14	12.6 ±0.337	14	12.7 ±0.216	20	12.3 ±0.201	19	11.8 ±0.199	20	12.1 ±0.269	18	12.4 ±0.189
Slice 12	13	10.7 ±0.120	14	12.2 ±0.324	14	12.4 ±0.211	20	11.8 ±0.204	19	11.3 ±0.209	20	11.6 ±0.262	19	11.8 ±0.206
Slice 11	13	10.5 ±0.120	13	11.9 ±0.330	14	12.1 ±0.195	20	11.4 ±0.191	19	11.0 ±0.237	19	11.0 ±0.207	19	11.3 ±0.197
Slice 10	12	10.3 ±0.118	14	11.7 ±0.335	14	11.9 ±0.218	20	11.1 ±0.193	19	10.6 ±0.218	20	10.8 ±0.238	14	11.0 ±0.088
Slice 9	16	10.8 ±0.256	14	11.5 ±0.321	14	11.9 ±0.244	20	10.9 ±0.170	19	10.5 ±0.219	20	10.6 ±0.215	14	10.7 ±0.097
Slice 8	16	11.0 ±0.305	14	11.9 ±0.328	14	12.2 ±0.264	20	10.7 ±0.166	19	10.4 ±0.214	17	10.2 ±0.150	19	10.7 ±0.217
Slice 7	16	11.7 ±0.330	12	13.2 ±0.297	14	13.1 ±1.21	20	11.0 ±0.179	19	10.9 ±0.223	19	10.7 ±0.196	19	11.0 ±0.234
Slice 6	16	13.3 ±0.435	14	14.3 ±0.467	14	14.5 ±1.56	19	12.0 ±0.196	18	12.1 ±0.263	20	12.0 ±0.249	20	12.4 ±0.350
Slice 5	16	16.1 ±0.524	14	16.9 ±0.713	14	17.2 ±1.82	20	14.6 ±0.272	19	14.6 ±0.292	20	14.4 ±0.345	20	15.0 ±0.497

SHAM = sham operated rats, OVX = non-milk ovariectomized rats, OVX ALD = non-milk ovariectomized + alendronate rats, GOAT OVX = goat ovariectomized rats, GOAT OVX ALD = goat ovariectomized + alendronate rats, COW OVX = cow ovariectomized rats, COW OVX ALD = cow ovariectomized + alendronate rats. Non-milk represents the rats fed the non-milk diet, Cow represents the rats fed the cow's milk based diet, and Goat represents the rats fed the goat milk based diet.

6. The effects of formulated goat milk and cow's milk diets with and without sodium alendronate on toughness of different regions of the femoral shaft of ovariectomized rats.

Bone fractures can occur as a consequence of osteoporosis in postmenopausal women. These fractures are most often caused by a sudden impact resulting from a fall. This study further investigates and compares the effects of the diets with and without Alendronate on the toughness of different regions within the femoral shaft of ovariectomized rats. Particularly this chapter seeks to examine whether the varying morphological changes found in chapter 4, within and between the rat groups fed either of the milk diets, resulted in tougher or brittle bone.

6.1. Introduction

The toughness of a material quantifies its ability to resist the propagation of a crack through its structure (1). Several different methods are available for measuring toughness in bone. Traditionally one of the more common measurements is the 'toughness of modulus' also known as the 'work to fracture', this is defined as the amount of energy used to fracture an object, and is most often explained as the area under the stress-strain curve (2). More popular methods used today use notched milled specimens of bone, and measure 'fracture toughness' or resistance to crack propagation (3). However, these methods are best suited to animals with large bones, such as humans, bovine animals or dogs. The preparation of samples involves cutting sections usually 1-2 centimetres in width and length from one side of a bone. The bones of the rat skeleton are simply too small to meet the requirements for milling adequate bone samples (4). In response to this, Ritchie *et al* (2008) developed and tested a method by which the femur was notched in the mid-shaft and then subjected to lateral bending in a three point bending test (4). This test allowed for the more complex fracture toughness measurements to be introduced to small animal toughness testing. Unfortunately this method was designed to test the whole bone, and could not easily be altered for testing the different regions within the femur that were being investigated in the current study. The main reason for this was that three point bending tests require the length of the bone sample to be at least 16 times the thickness of the sample (5). If the ratio between length and thickness is too small, then the loading forces the bone sample will be subjected to will be largely shear forces and not bending forces resulting in inaccurate data (6). Technically it is already understood that subjecting the entire rat femur to three point bending tests already breaks this rule, and introduces shear stresses and as a result increased error (1). In light of this it was decided not to use the method described by Ritchie *et al* (2008) (4), and three point bending was removed as an option for testing the toughness of the segments within the femoral shaft.

The method developed for this study used the engineering principles of a suddenly applied impact force often used in materials testing. This is more commonly known as an 'Izod test' and uses a suddenly applied force to impact the samples. The decision to use a sudden impact force rather than an statically applied force was because most real life bone fracture injuries are as a result of a sudden impact, i.e. a fall (7, 8).

Bisphosphonates such as Alendronate halt bone resorption and slow bone turnover, thereby decreasing the risk of osteoporotic fracture by generally improving mineralization (9, 10). However, the use of anti-resorptive drugs to suppress remodelling have also been shown to accumulate micro damage in the bone tissue, thereby reducing the bone's ability to absorb energy and increases bone brittleness (11, 12). The aim of this experiment was to determine the effect of diet with and without sodium Alendronate on the toughness of different sections of the femoral shaft in ovariectomized rats.

6.2. Method & materials

Right femurs were collected from the rats used in the ovariectomized rat trial outlined in chapter 4. The femurs were removed from euthanized animals by simple dissection, and placed in phosphate buffered saline (PBS) at -20° Celsius pending further analysis.

6.2.1. Summary of groups

As described in chapter 5, one hundred and forty Sprague-Dawley female rats were randomly divided into seven groups (Table 6-1). The rats were divided into three groups as weanlings (3 weeks of age) and fed, the non-milk diet (60 rats), goat milk diet (40 rats) or cow's milk diet (40 rats). At 22 weeks of age the rats underwent ovariectomy or sham-ovariectomy surgery. The rats fed the non-milk diet were divided into two groups, sham operated (SHAM) and ovariectomized (OVX); all rats fed the milk based diets were ovariectomized. At 30 weeks of age an Alendronate (ALD) drug treatment was introduced to 20 randomly selected ovariectomized rats from each diet group. The remaining 20 ovariectomized rats from each diet group and the SHAM rats were given a placebo treatment.

Table 6-1. The table shows the seven treatment groups in the mature OVX rat trial.

Diet	Non-milk			Cow		Goat	
Treatment Group	Sham	Ovx	Ovx Ald	Ovx	Ovx Ald	Ovx	Ovx Ald
Rats per group	20	20	20	20	20	20	20

The rats were fed, the non-milk diet (60 rats), a goat milk based diet (40 rats) or a cow's milk based diet (40 rats). The non-milk rats underwent either ovariectomy surgery (OVX) or sham-ovariectomy surgery (SHAM) all other groups were ovariectomized. At 30 weeks of age the Alendronate (ALD) drug treatment was introduced to the diet Non-milk group (OVX ALD), the Goat milk diet group (GOAT OVX ALD), and the Cow's milk diet group (COW OVX ALD). The remaining 20 ovariectomized rats from each diet group were given a placebo, Non-milk diet (OVX), Goat milk diet (GOAT OVX), and Cow's milk diet (COW OVX). The sham operated rats were also given the placebo.

6.2.2. Femur sections

The right femurs were thawed, and their weight and length determined after any residual adherent soft tissue had been removed. The measured length of each femur was then divided into five segments marked at 25%, 45%, 65% and 85% of the total length. Each segment was then sectioned along the transverse lines with a diamond wheel saw. These segments spanned 60% of the overall length, and extended from the upper limit of the intercondylar ridge (segment C) at the distal end of the femoral shaft, to the upper limit of the third trochanter (segment A) at the proximal end of the femoral shaft. Each section was marked at 50% of its length and stored in PBS at 4° C. All segments were checked to ensure that they had not been damaged during cutting. Any cracked segments were discarded.

6.2.3. Izod test

The Izod test is an impact test traditionally used in engineering as a comparative tool to measure the resistance to failure of a material to a suddenly applied force (13). This is expressed as impact energy, or the amount of energy needed to fracture the test sample. The pendulum has two types of energy - potential and kinetic. The maximum potential energy is stored at the start of the swing and converts to maximum kinetic energy at the lowest point of the swing (Fig. 6-1). The striker on the pendulum impacts the bone sample at the moment of maximum kinetic energy, at this point the energy is absorbed by the bone until fractures occurs. The pendulum then continues on into an upward swing. The impact energy (Joules) is calculated by the loss of energy in the pendulum swing after it strikes the bone sample (7, 13). The difference represents how much

energy was absorbed by the bone sample before it fractured. A tougher bone sample will absorb more energy before fracturing compared to a brittle bone sample, which will fracture with less energy. The specific energy (Joules/mm^2) of a material is the amount of energy per unit of area required to fracture that material.

The above scenario assumes that the pendulum pivot is frictionless and there is no air resistance on the striker as it swings. Therefore, to reduce any possible error that these assumptions make, the pendulum was let freefall 10 times before testing began, and after every ten bone segments tested. The height of the pendulum was recorded after every freefall swing and the average energy lost by the pendulum was subtracted off the results of each segment.

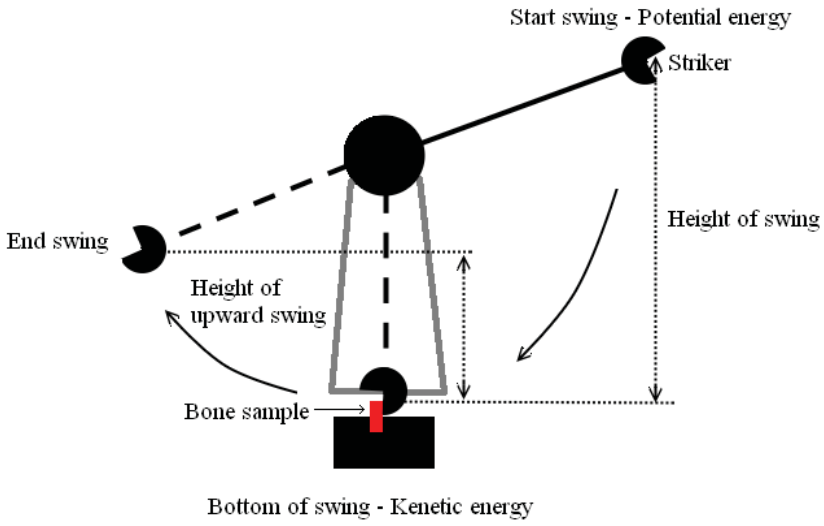
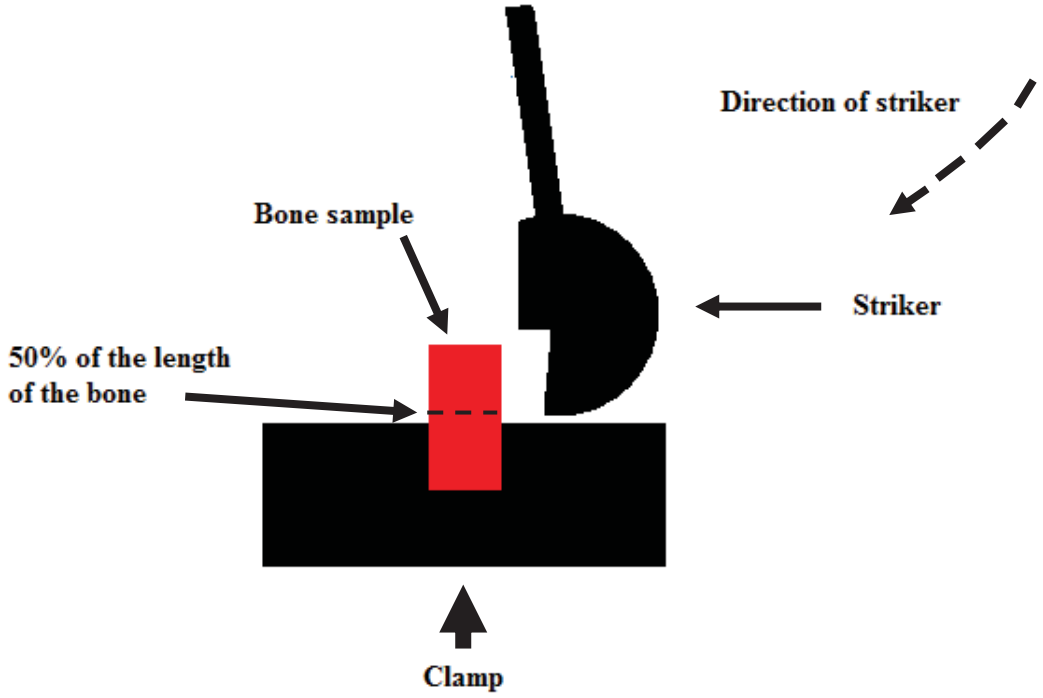


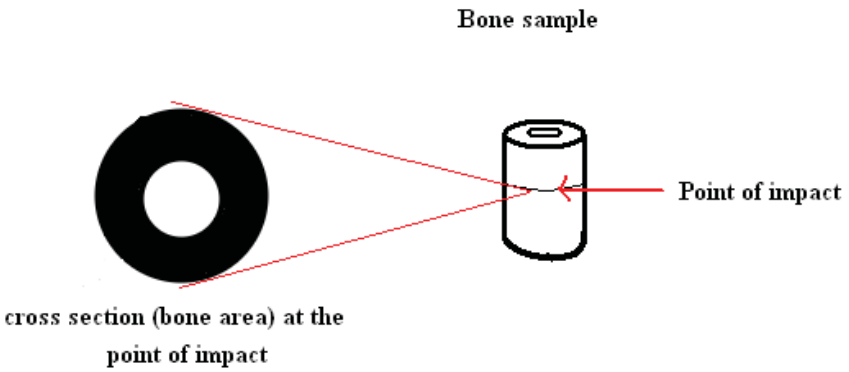
Figure 6-1. Diagram of Izod test.

The biomechanical testing was done using a "Zwich" impact testing machine (Zwick/Roell, Germany). Each bone sample was gripped firmly at the distal end of the segment, leaving the mid and proximal portions of the segment exposed (Fig. 6-2a). The bone samples were gripped at a height to ensure that the striker impacted the bone segment consistently at 50% the length of the sample. All segments were held under the same orientation to reduce error, and kept wet throughout the test.

The pendulum had 0.5 Joules of potential energy at the start of each swing. It was held at a 160° angle and let freefall until it hit the sample. Upon impact the bone segment was broken completely into several pieces. The impact energy (J) lost from the pendulum to the bone segment across the bone segments cross section (mm2) was recorded and expressed as energy absorbed per unit of bone area (J/mm^2) (8). This is the material's specific energy. The bone area measurement was taken at the point of impact across the transverse cross section of the bone (Fig. 6-2b). The bone area of the samples could not be taken directly from the bone segments broken in the impact test, as they were too damaged to accurately determine the area. Therefore the measurements were taken from the left femur bone area measurements, under the assumption that differences in bone size and bone area between left and right femurs are not significant (14).



a) Diagram showing bone sample clamped in tester.



b) Diagram of bone sample showing the point of impact between the pendulum and the bone segment, with the impact occurring half way up the length of the segment. The cross section shows the area of the bone used to calculate the bone area (mm^2) measurement.

Figure 6-2. Diagrams showing orientation of bone samples in Izod tester.

6.2.4. Statistical analysis

It was decided to run a separate analysis of the rats fed the non-milk diet from the rats fed the milk diets. A first attempt to run the data together failed as the data from the non-milk groups prevented the data from normalizing. Therefore the analysis of the milk diets, GOAT OVX, GOAT OVX ALD, COW OVX and COW OVX ALD were run as a separate series of analyses to the rats on the non-milk diet (SHAM, OVX and OVX ALD).

Data were analysed in the statistical package "SYSTAT" (version 11) (Systat, Chicago, USA). Statistical significance was set at $p<0.05$. Biomechanical measurements were found for the most part to be normally distributed for the segments. Where non-normal data were found a log transformation was required to obtain near normal distribution on graphic analysis. Impact energy (J) for segments A and B were transformed using a Johnson transformation (15), as was bone area (mm^2) for segments A and B with the statistical package "MINITAB" (Minitab Inc, Pennsylvania, USA). Outliers were removed from some groups to allow for data to be transformed to a normal distribution. The number of outliers removed never exceeded 10% of the group number. The data for segments A and B were then transformed using

$$Y = 1.198 + 0.950 * [\ln(x-0.23) / (0.214-x)]$$

Similarly bone areas from segments A and B were transformed using

$$Y = 0.325 + 0.958 * [\ln(x-5.681) / (10.60-x)]$$

Specific energy (J/mm^2) for segment C data, and Impact energy (J), could not be transformed to represent a normal distribution and all data for this segment were therefore excluded from further analysis. This was likely a result of extensive in-homogeneity of transverse slices in the segment.

Groups fed the Goat or Cow's milk diets

The variation of impact energy, with treatments was assessed by 2-way ANOVA. The relationships between the impact energy (J) used to break the bone samples and the bone area (mm^2), and total cross sectional area (mm^2) of the proximal and mid-segments were assessed by ANCOVA's and linear regressions. Student's T tests were used to compare the slope of the linear regression and were calculated by,

$$T = (a_1-a_2)/SE_{a1-a2}$$

where, a_1 = mean slope of the ovariectomized + Alendronate treatment group and a^2= mean slope of the ovariectomized group. SE = standard error of the comparison, and was calculated using

$$SE_{a1-a2} = \sqrt{SE_{a1}^2 - SE_{a2}^2} \quad (16).$$

Statistical significance of T was intercalated from the students T table.

The mean overall bone area was calculated from the combined areas of the four slices of the left femur for each of the segments A and B. This was repeated for overall cross sectional area. The variation of bone area and overall cross sectional area, with treatments was assessed by 2-way ANOVA.

Groups fed the Non-milk diet

The impact energy of the three groups of rats fed the non-milk diet was examined by one-way ANOVA. The relationships between the impact energy (J) used to break the bone samples and the bone area (mm^2), and total cross sectional area (mm^2) of the proximal and mid-segments were assessed by ANCOVA's and linear regressions. Student's T tests were used to compare the slope of the linear regression (see above for equations).

The mean overall bone area was calculated from the combined areas of the four slices of the left femur for each of the segments A and B. The variation of bone area and overall cross sectional area, with treatments was assessed by one-way ANOVA and significant differences identified where examined using Tukey post-hoc testing.

6.3. Results

6.3.1. Milk diets – Segment A

Specific energy

The specific energy required to fracture the femoral segments A of OVX ALD rats were significantly greater ($p<0.05$) than the OVX rats (Fig. 6-3). The specific energy required to fracture segments A of the femurs of rats that received COW OVX ALD were not significantly different to those required to fracture those of rats which received GOAT OVX ALD. i.e. there was no significant interaction between milk and drug.

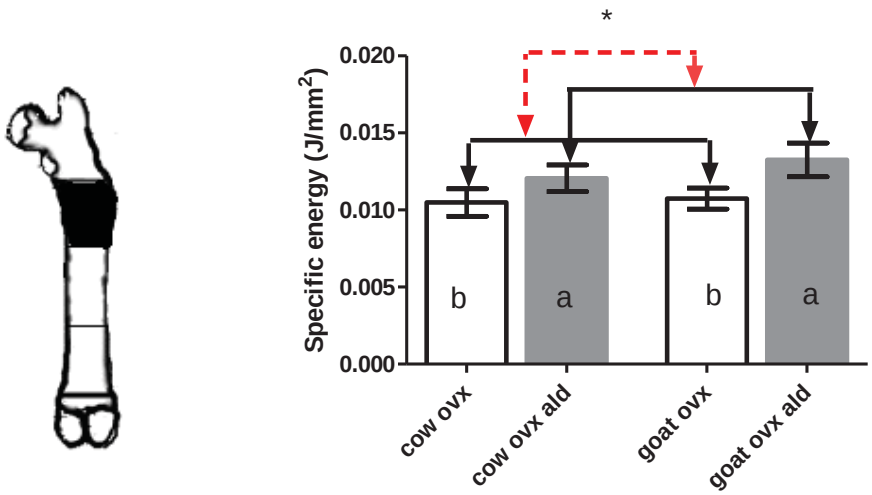


Figure 6-3. Means and SD's of specific energy (J/mm^2) required to fracture segment A for rats fed milk diets.

* indicates a significance of $p<0.05$. Values with different letters are significantly different using 2-way ANOVA. Specific energy is the energy required to fracture the bone sample / bone area of bone sample at the point of impact (see methods for description). Cow ovx = ovariectomized rats that received cow's milk diet (n= 18), cow ovx ald = ovariectomized rats that received cow's milk diet and Alendronate (n= 18), goat ovx = ovariectomized rats that received goat milk diet (n= 19), goat ovx ald = ovariectomized rats that received goat milk diet and Alendronate (n= 19).

Morphology – Bone area and Total cross sectional area

There was no significant difference on 2-way ANOVA between the bone areas of femoral segments A of OVX ALD rats and the OVX rats. However, the bone areas of the femoral segments A of GOAT OVX rats were significantly larger ($p<0.05$) than the COW OVX rats (Fig. 6-4a).

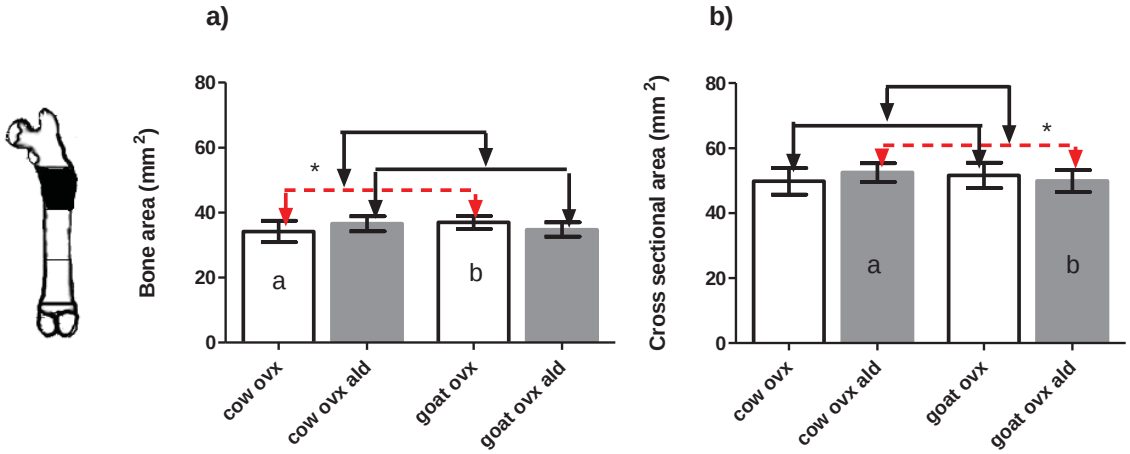


Figure 6-4. Means and SD's of a) bone areas (mm^2) and b) cross sectional areas (mm^2) of femoral segments A for rats that received milk diets.

* indicates a significance of $p<0.05$. Values with different letters are significantly different using 2-way ANOVA. COW OVX = ovariectomized rats that received cow's milk diet (n= 20), COW OVX ALD = ovariectomized rats that received cow's milk diet and Alendronate (n= 20), GOAT OVX = ovariectomized rats that received goat milk diet (n= 20), GOAT OVX ALD = ovariectomized rats that received goat milk diet and Alendronate (n= 19).

There were no significant differences between the total cross sectional areas of femoral segments A of OVX ALD rats and the OVX rats. The total cross sectional areas of femoral segments A were significantly larger in COW OVX ALD rats than in the GOAT OVX ALD ($P<0.05$) i.e. there was significant interaction between milk and drug (Fig. 6-4b).

Relationship between impact energy and bone area, and total cross sectional area

The effect of drug and diet on cross sectional areas (bone size), bone areas and impact energy was explored by ANCOVA. Neither the size, nor the bone area of femoral segments A appeared to influence the impact energy required to break them in OVX rats.

6.3.2. Milk diets – Segment B

Specific energy

There were no significant differences on two-way ANOVA between the specific energy required to fracture the femoral segments B of the OVX ALD rats and the OVX rats. However, the femoral segments B of the GOAT OVX ALD rats required somewhat greater specific energy to fracture ($p<0.073$) than the COW OVX ALD rats (Fig. 6-5).

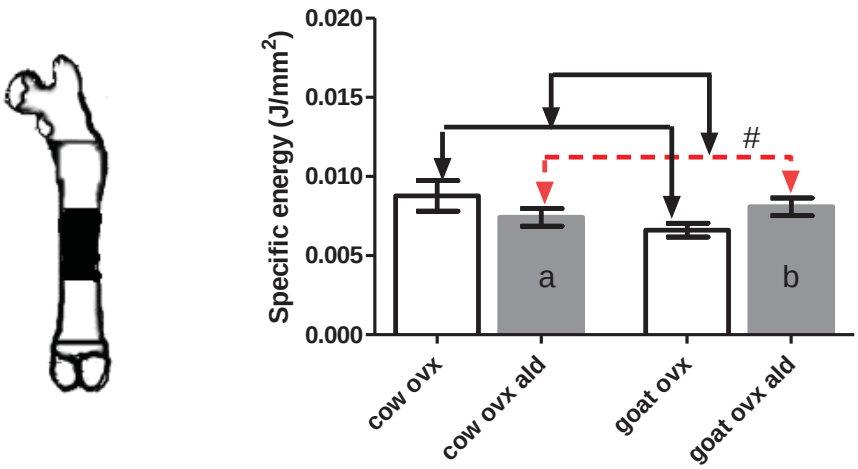


Figure 6-5. Means and SD's of specific energy (J/mm^2) required to fracture segment B for rats that received milk diets.

Impact energy is the energy required to fracture the bone sample / bone area of bone sample at the point of impact (see methods for description). * indicates a significance of $p<0.05$, however there was a tendency towards significance at ($p<0.073$) and was indicated by '#'. Values with different letters are significantly different using 2-way ANOVA. Cow ovx = ovariectomized rats that received cow's milk diet (n= 18), cow ovx ald = ovariectomized rats that received cow's milk diet and Alendronate (n= 18), goat ovx = ovariectomized rats that received goat milk diet (n= 17), goat ovx ald = ovariectomized rats that received goat milk diet and Alendronate (n= 17).

Morphology – Bone area and Total cross sectional area

There were no significant differences between the bone areas of femoral segments B of OVX ALD rats and the OVX rats. The bone areas of the segments B of COW OVX ALD were significantly greater ($p<0.05$) than the GOAT OVX ALD rats, i.e. there was a significant interaction between milk and drug (Fig. 6-6a).

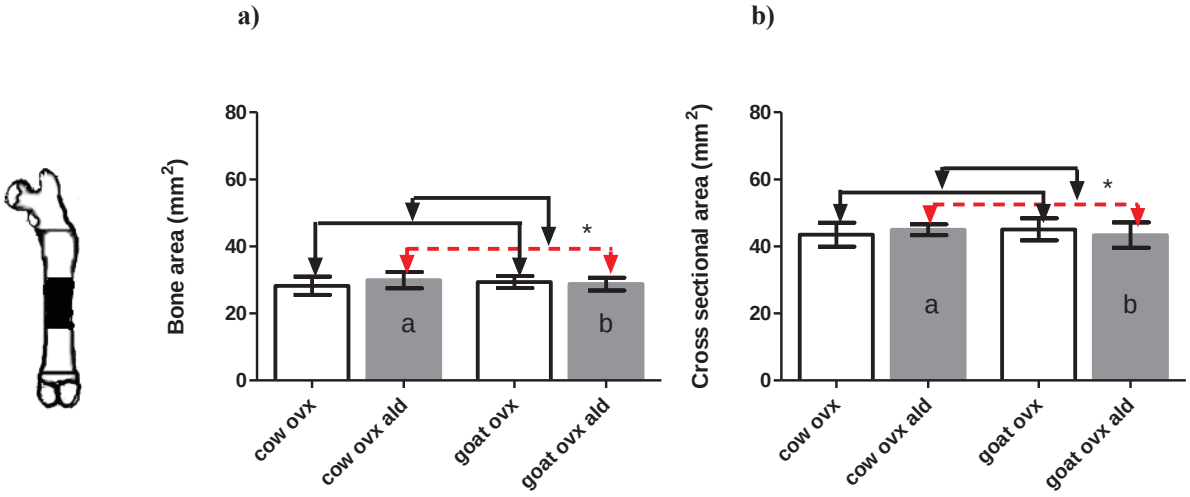


Figure 6-6. Means and SD's of a) bone areas (mm^2) and b) cross sectional areas (mm^2) in femoral segment B for rats that received milk diets.

* indicates a significance of $p<0.05$. Values with different letters are significantly different using 2-way ANOVA. Cow ovx = ovariectomized rats that received cow's milk diet (n= 20), cow ovx ald = ovariectomized rats that received cow's milk diet and Alendronate (n= 20), goat ovx = ovariectomized rats that received goat milk diet (n= 20), goat ovx ald = ovariectomized rats that received goat milk diet and Alendronate (n= 19).

There were no significant differences between the cross sectional areas of femoral segments B of the OVX ALD rats and the OVX rats. The cross sectional areas of femoral segments B of COW OVX ALD rats were significantly greater ($p<0.05$) than the GOAT OVX ALD i.e. there was a significant interaction between milk and drug (Fig. 6-6b).

Relationship between fracture energy and bone area, and total cross sectional area

ANCOVA indicated that the mean levels of impact energy (J) required to fracture the femoral segments B of GOAT OVX ALD rats was significantly higher ($p<0.05$) than that of the GOAT OVX rats. The rate of increase in impact energy required to fracture femoral segments B with an increase in bone area, was significantly greater in GOAT OVX ALD rats ($P<0.05$) compared to the GOAT OVX rats. This suggests an interaction between drug and bone area. The slope coefficients obtained from linear regressions also showed that the GOAT OVX ALD rats were significantly different to the GOAT OVX rats on Student's T test ($p<0.05$).

A similar comparison conducted on COW OVX rats with and without ALD showed no significant relationship between bone area and impact energy, nor any effect of ALD on this relationship. Therefore the GOAT OVX ALD rats had smaller and tougher bone in the shafts of their femurs than the COW OVX ALD rats. The COW OVX ALD rats may have grown greater quantities of weaker bone.

ANCOVA failed to demonstrate any significant relationship between impact energy required to break segment B and the cross sectional area of this segment. Nor was there any effect of ALD on this relationship either in GOAT OVX rats or COW OVX rats.

In summary the administration of goat milk with Alendronate caused the mid shaft of the femur to have greater toughness per unit of bone area than when cow's milk was administered with Alendronate. Therefore, the relative increase in the toughness of the mid-shaft where goat milk and Alendronate rather than cow's milk and Alendronate was administered, resulted from an increase in the transverse toughness rather than the size of the bone.

6.3.3. Non-milk diet – Segment A

The data from the rats fed the non-milk diet were excluded from the other group analyses due to skewing of results. Therefore, the effects of the non-milk diet SHAM operated rats OVX rats and OVX ALD rats on specific energy in the proximal region (segment A) of the femoral shaft were investigated by one way ANOVA with Tukeys post hoc testing. The specific energy required to fracture the femoral segments A were not significantly different between any of the treatments (Fig. 6-7).

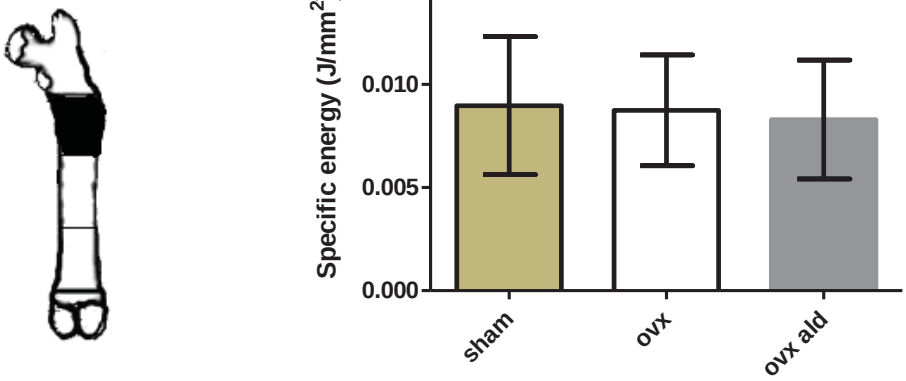


Figure 6-7. Means and SD's of specific energy (J/mm^2) required to fracture segment A for rats that received the non-milk diet.

Specific energy is the energy required to fracture the bone sample / bone area of bone sample at the point of impact (see methods for description). Values with different letters are significantly different, $p<0.05$. Sham = sham operated rats (n= 15), ovx = ovariectomized rats(n= 13), ovx ald = ovariectomized rats that received Alendronate (n= 14).

Morphology and treatment – Bone area and Total cross sectional area

OVX ALD rats had significantly greater bone areas ($p<0.05$) compared to the SHAM rats, and the OVX rats were not significantly different to either group in bone area (Fig. 5.8a). In contrast, the OVX and OVX ALD rats had significantly greater total cross sectional bone areas ($p<0.05$) compared to the SHAM rats (Fig. 6-8b).

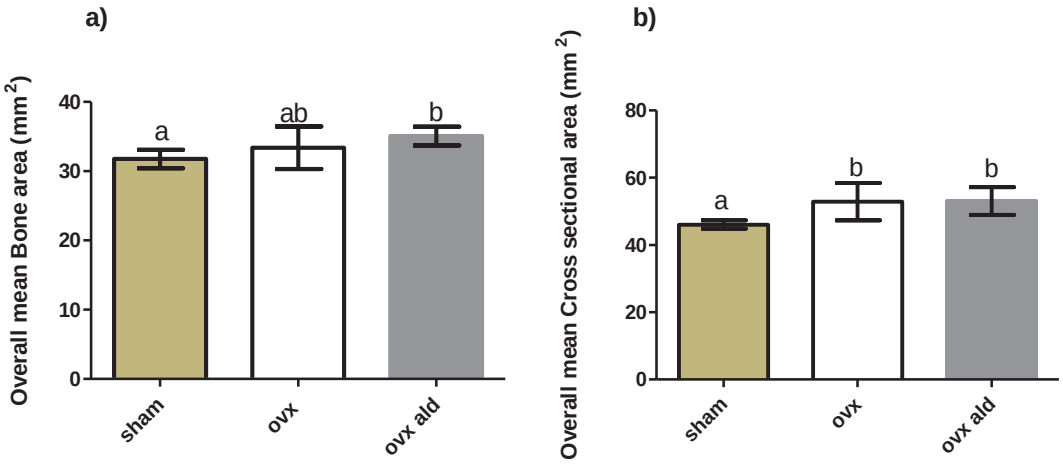


Figure 6-8. Means and SD's of a) bone areas (mm^2) and b) cross sectional areas (mm^2) in femoral segment B for rats that received the non-milk diet.

Values with different letters are significantly different, $p<0.05$. Sham = sham operated rats (n= 16), ovx = ovariectomized rats (n= 14), ovx ald = ovariectomized rats that received Alendronate (n= 14).

Relationship between fracture energy and bone area

ANCOVA failed to demonstrate any significant relationship between the impact energy required to fracture segment A, and the bone area of this segment. Nor was there any effect of treatment on this relationship.

ANCOVA indicated a significant relationship between impact energy and total cross sectional area. It showed the mean levels of impact energy required to fracture the femoral segments A of the OVX rats was significantly higher ($p<0.05$) than that of the OVX ALD rats. The rate of increase in impact energy required to fracture femoral segments A with an increase in total cross sectional area, was significantly greater in OVX rats ($p<0.05$) compared to the OVX ALD rats. This suggests an interaction between treatment and total cross sectional area. The slope coefficients obtained from linear regressions also showed that the OVX rats were significantly different to the

OVX ALD rats on Student's T test ($p<0.05$). There was no significant difference between the ovariectomized rats, and the SHAM rats.

In summary there was a significant relationship between the impact energy needed to break the femoral segments A and bone size. However, it was not enough to influence the toughness of the bone in relation to a sudden impact.

6.3.4. Non-milk diet – Segment B

One way ANOVA found that the specific energy required to fracture the femoral segments B were significantly greater for OVX rats ($p<0.05$) compared to OVX ALD rats (Fig. 5.9). SHAM operated rats ($p<0.064$) had a tendency to require greater energies before fracture compared to OVX ALD rats.

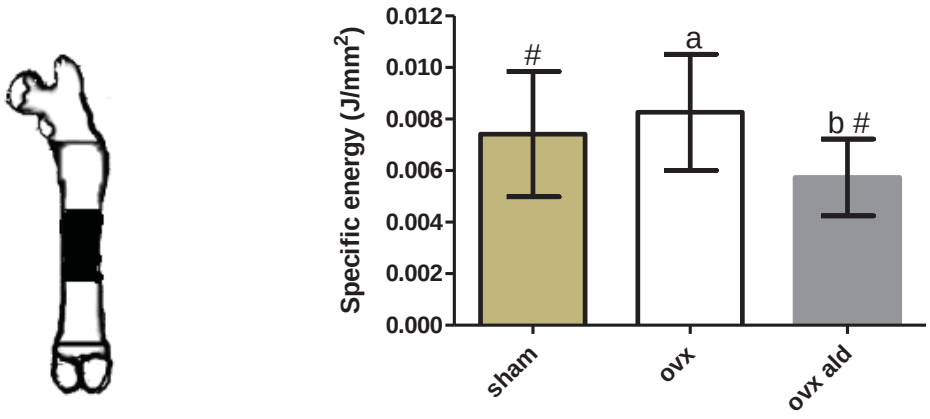


Figure 6-9. Means and SD's of specific energy (J/mm^2) required to fracture segment B for rats that received the non-milk diet.

Specific energy is the energy required to fracture the bone sample / bone area of bone sample at the point of impact (see methods for description). Values with different letters are significantly different, $p<0.05$, however there was a tendency towards significance between Sham and ovx ald at ($p<0.064$) and was indicated by'#'. Sham = sham operated rats (n= 14), ovx = ovariectomized rats (n= 12), ovx ald = ovariectomized rats that received Alendronate (n= 14).

Morphology and treatment - Bone area and Total cross sectional area

OVX ALD and OVX rats had significantly greater bone areas both ($p<0.05$) compared to the SHAM rats (Fig. 6-10a). The OVX ALD and OVX rats also had significantly greater total cross sectional bone areas ($p<0.05$) compared to the SHAM rats (Fig. 6-10b).

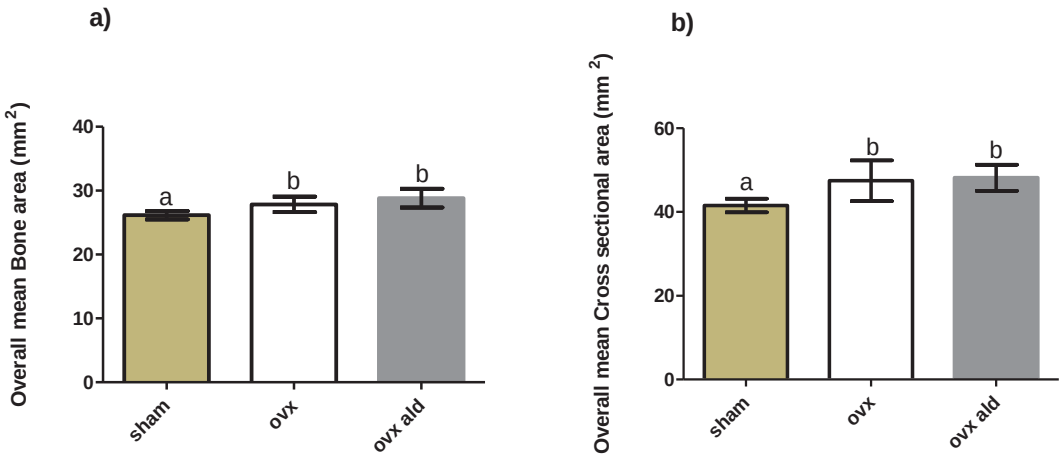


Figure 6-10. Means and SD's of a) bone areas (mm^2) and b) cross sectional areas (mm^2) in femoral segment B for rats that received the non-milk diet.

Values with different letters are significantly different, $p<0.05$. Sham = sham operated rats (n= 16), ovx = ovariectomized rats (n= 14), ovx ald = ovariectomized rats that received Alendronate (n= 14).

Relationship between fracture energy, total cross sectional area and bone area

ANCOVA showed a significant relationship between the impact energy needed to fracture femoral segments B and the CSA ($p<0.05$), but there was no significant relationship between impact energy and bone area, nor was there any effect of treatment on this relationship.

In summary the OVX and SHAM rats had tougher bones than the OVX ALD rats. The larger total cross sectional area of the OVX group may have had a positive effect of the toughness of the femoral segment B. However, the larger bone sizes found in the OVX ALD was not sufficient to increase the toughness to the levels found in the OVX and SHAM rats.

6.4. Discussion

6.4.1. Testing method

The small size of the bone samples in the current study precluded the use of existing 'fracture toughness' testing methods. These methods use larger animal bone samples such as bovine or human and are milled from cortical bone to sizes of approximately 35 to 45 mm in length with a width and depth between 2 – 4 mm (17-19). The only known 'fracture toughness' testing method for small animal is described in Ritchie *et al* (2008), where a 1μm notch was cut into posterior of the femur mid-shaft, and then the entire femur subjected to a three-point bending test with the notch facing downward (4). Ritchie *et* al (2008) described the method as an effective tool in measuring the behaviour of crack propagation in small animal bone with the linear-elastic fracture mechanics measurement K_c offering the most reproducible and accurate results (4). The decision to use the un-notched impact test to investigate the toughness of the bone segments in this study over the 'fracture toughness' method, was the ability to apply the impact test to more than one region in the femoral shaft. This decision allowed for the continuation of investigating the regional variations within the femoral shaft of rats with different feeding and drug regimes.

There are both advantages and disadvantages in using an un-notched method. The preparation of the un-notched samples is less likely to introduce flaws or new cracks into the bone samples compared to notched milled samples, however, as the bone samples become smaller both notched and un-notched methods increase the possibility of introducing flaws as the samples are prepared. Disadvantages in using an un-notched method is the presence of pre-existing micro-cracks within the bone material that may pre-weaken the bone and increase error within the results (4, 20, 21). This may explain the large spread of data Currey (1979) reported when subjecting un-notched human bone specimens to a sudden impact from a Hounsfield plastics impact tester (8). Currey (1979) did point out that increased variation in results was not uncommon when using this type of testing method (8). The un-notched impact test used in the current trial is of limited use in that it did not describe the behaviour of the applied energy on the fracture; however, it was sensitive enough to describe biomechanical variations within the femoral shaft (as discussed below).

6.4.2. The effect of diet and drug on impact energy

To date we have shown that the milk diets had a positive effect on overall bone mass and morphology in the ovariectomized rat model for osteoporosis. The principle aim of this study was to determine whether there was a complementary effect between the diets supplemented with milk and the administered Alendronate on the specific energy needed to fracture bone. The proximal segment of the femoral shaft did show that the rats fed the milk diets and dosed with Alendronate had significantly tougher bone material than the rats fed the milk diets and given the placebo. This suggests that Alendronate had a positive response with both milk diets. Yet there was no significant difference when looking at treatment effect within the rats fed the non-milk. It is unclear as to why there was a complementary effect between the drug and the milk diets, and no drug response in the rats fed the non-milk diets. However previous research has shown equally inconstant results in dogs dosed with Alendronate over a three year period. In one trial the dogs were treated with the Alendronate therapy of 1mg/kg/day and showed compromised toughness in the cortical bone of the rib cage (22), whereas the other trial with a dose rate of 0.6mg/kg/day found no significant differences between treatments in the rib cages of dogs (23). The reasons for these similar trials giving conflicting results is unclear and may be due to the differing methods used to determine toughness in the bone samples or possibly the difference in dose rates of the drug. Studies have shown that dogs given a lower dose rate of 0.2mg/kg/day have not shown significant differences in toughness where higher dose rates of 1mg/kg/day have compromised bone toughness (24). Whatever the case the results of the current study may simply be an effect of the vitamin A deficiency interfering with the Alendronate drug that was discussed in chapter 5; or possibly evidence of the complementary effect of the co-administration of milk diet and the drug therapy.

Unlike the proximal region, segment B of the rats fed the non-milk diet did show a significant difference between groups. It suggested that the bone material of the mid-shaft of the CON OVX ALD rats required less specific energy per unit of bone area to fracture than the CON OVX rats ($p<0.05$) and possibly the SHAM rats ($p<0.064$). This again conflicts with other literature where a study in dogs given a dose rate of

1mg/kg/day, found that there was no significant effect of Alendronate use on bone toughness in the femoral mid-shaft (25).

6.4.3. Regional variation of impact energy

Bone is an anisotropic material; therefore, its toughness varies according to the direction in which the disrupting force is applied. The current study used a sudden impact force applied transversely to the long axis of the femur, which can be viewed as an analogous situation to that found in real life. However, the toughness of the femur varies not only in the direction of the applied force as it impacts the side of the femur, but also with the location at which the force is applied. Regional variations in toughness in the femurs of humans were investigated by Brown *et al* (2000), who showed that the neck was tougher than the shaft under both tension and shear loading (26). The current study found that there were no regional differences between SHAM and CON OVX rats suggesting that ovariectomy surgery did not impact on the toughness of the middle and upper femoral shaft. However there was a tendency for the GOAT OVX ALD rats to have tougher bone material per unit of area than the COW OVX ALD rats in the mid-shaft of the femur ($p<0.073$). In the proximal region of the rats fed the milk diets, the toughness of the bone material were not determined by the milk diets but the combination of both milk diet and alendronate.

6.4.4. Effect of morphology on the energy needed to break segments

Localized toughness testing allowed for the examination of the relationship between bone area and the amount of energy (J) needed to break the femoral segments. The current work showed that there was a significant relationship between bone area and the amount of energy needed to fracture segment B in the rats that received GOAT OVX ALD. In contrast, no relationship was found between bone area and energy needed to fracture segment B in rats that received cow's milk either with or without Alendronate. No overall relationship was found between total cross sectional area (an index of structural size) and the amount of energy needed to fracture femoral segment B for rats that received either of the milk diets with or without alendronate. Taken together the results from bone area and total cross sectional area suggest that the increase in the amount of energy needed to fracture segment B of GOAT OVX ALD rats is due to changes in the toughness of the bone material, and not the cross sectional size of the femur. Therefore, the smaller bone areas of the femoral segments B of GOAT OVX

ALD rats were perhaps tougher than the larger sized bone segments of the COW OVX ALD rats. The effect of morphology on the behaviour of fracture propagation has been examined in the micro-structure of human cortical bone by Yeni *et al* (1997) (27). They found that osteon morphology (both size and density) and the degree of cortical bone porosity accounted for 49% - 68% of the variation in fracture toughness of milled notched samples taken from expired elderly human femurs and tibias (27). However, on the macro scale, previous work has suggested that the fracture properties, K_c and G_c are not influenced by morphological properties such as bone thickness (28, 29). It remains unclear in the current study as to why the GOAT OVX ALD rats had smaller bone areas compared to the COW OVX ALD rats. It may be that the alendronate reduced periosteal apposition and at the same time allowed for a more complete mineralisation of the bone composite compared to the COW OVX ALD rats. In contrast further investigation of the rats fed the non-milk diet showed that there was a relationship between the size of the bone and the energy used to fracture the bones in the proximal femoral shaft of the CON OVX rats. While it did not result in tougher bones it did indicate that, as the size of the proximal shaft in the CON OVX rats got bigger, more energy was needed to break the bone segments. This was also apparent in the mid-shaft of CON OVX rats, except that the relationship between impact energy and bone size was reflected in significantly tougher bone compared to the CON OVX ALD rats. There was some difference in toughness between the SHAM rats and the CON OVX ALD rats in the mid-shaft. However, that difference may have been offset by the larger sized bones found in the CON OVX ALD rats.

6.4.5. Conclusion

A complementary effect was found between the milk based diets and alendronate in the proximal region of the femoral shaft resulting in tougher bone material per unit of area. The co-administration of goat milk and alendronate appeared to have a complementary effect in increasing the toughness of the bone composite in the mid sections of the femurs of 10 month old ovariectomized rats. The localisation of this effect in a limited region of the femur emphasises the importance of evaluating regional variation of toughness within the femur, when assessing the effect of dietary and medical therapies in animal models of osteoporosis. It is uncertain why supplementation with alendronate caused a different response when rats were fed goat's milk compared to cow's milk, and further investigation would be required.

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7. Effect of a formulated goat milk diet on trabecular bone in the tibia using micro-CT in ovariectomized rats.

The development of μCT technology has allowed for the detailed investigation of the trabecular regions of bone, and has established itself as a method of investigation in pre-clinical osteoporosis research. Trabecular bone is more sensitive than cortical bone to the effects of osteoporosis and to the effects of the drug alendronate in arresting bone resorption. This study compared the effects of goat milk and goat milk with the administration of alendronate on architectural changes the trabecular region of the proximal tibia.

7.1. Introduction

Trabecular bone serves as a reservoir for minerals and contributes to the overall biomechanical competence of the skeletal system. It is arranged by a complex interlacing network of trabeculae surrounded by a thin shell of compact cortical bone. Consequently, trabecular bone has a large surface area, which is ideally suited for absorbing and distributing stresses placed on it when the bone has loading forces directed upon it (1).

In women, trabecular osteoporotic bone loss for the most part is due to the complete removal of trabeculae from the structure resulting in increased open spaces (2). This degree of bone loss has also been seen in ovariectomized rats where the rapid loss of trabecular bone has been tracked early after ovariectomy surgery with up to 57% of trabecular bone lost within two months (3). This degradation, which is believed to be irreversible, compromises the structure by the loss of connectivity between trabeculae, this causes the trabecular region to become more fragile and increases the risk of fracture (4-6).

Traditional three-point bending strength tests are usually restricted to the mid-shaft of the femur or tibia. While they have provided insight into the effects of ovariectomy on the whole bone, the loads are applied primarily to regions that are made up of largely cortical bone. Trabecular bone is known to be more sensitive to the effects of osteoporosis in both humans and rats, therefore testing just cortical bone may not be sufficient to determine the mechanical competence of osteoporotic bone (7). Successful strength testing has been done on trabecular bone samples by machining small cubes of trabecular bone out of the vertebrae of the lumbar spine, the distal femur or the proximal tibia. However, the sample preparation for these tests can be difficult, particularly in small animals. Recently Sturmer *et al* (2006) developed a method of strength testing the largely trabecular region of the proximal tibia without having to cut sections from the whole bone (8). They selected the metaphyseal region of the tibia due to the reliability of showing a response to osteoporotic like conditions, and due to the reproducibility of fractures using their testing method (8).

No studies could be found that describes the effects of a goat milk diet on trabecular bone quality. Therefore the aim of this study was to investigate the effects of a goat milk based diet with or without alendronate on the trabecular architecture and strength of the tibias of female rats ovariectomized at 6 months of age.

7.2. Methods & materials

7.2.1. Bone samples

Tibias from the experiment conducted in Chapter 4 were thawed and any residual adherent soft tissue removed then stored in 70% ethanol. Then 10 left tibias from each treatment group were randomly selected and sent to Auckland University for μCT scanning. The right tibias from each group were set aside for biomechanical testing.

7.2.2. Treatment groups

The bone samples used in this study were taken from the rats raised as part of the study described in chapter 5. The groups used were the rats fed the goat milk diet + ovariectomized (GOAT OVX), and rats fed the goat milk diet + ovariectomized + Sodium alendronate (GOAT OVX ALD).

7.2.3. Micro-computed tomography

The left tibias were scanned by micro-computed tomography (μCT, using a Skyscan1172, Aartselaar, Belgium). Each sample was held under the same orientation and a 5.3mm length of the proximal region of the tibia was scanned. A total of 964 two-dimensional axial slices were taken and reconstructed into a three dimensional (3D) image of each tibia using the settings given in Table 7-1. These slices were binarized into a 3D image using global thresholding. The architectural parameters for the 3D trabecular and cortical were evaluated using custom analysis software (CTAn, Skyscan, Aartselaar, Belgium). The calculation methods used to determine the bone parameters are described on the Skyscan website (www.skyscan.be) and by Chappard *et al* (2001) (9).

Table 7-1. µCT operation settings

µCT Settings	
Source voltage (kV)	49
Current (µA)	200
Aluminium filter size (mm)	0.5
Isotropic voxel size (µm)	5

Settings used by Skyscan1172 µCT

For quantitative micro-structural analysis of trabecular bone, three-dimensional analysis was performed on a 301 slice volume of interest starting 30 slices distal to the proximal growth plate. The volume used for the cortical region was comprised of 101 slices, starting 476 slices from the growth plate. The trabecular and cortical volumes of interest were outlined by interpolation of operator-drawn regions exclusively representing trabecular and cortical bone respectively. All the work described in this method for µCT was carried out by staff at Auckland University.

The cortical bone properties were measured using bone volume mm^3 (BV), bone surface mm^2 (BS), Surface to volume ratio mm^{-1} (BS/BV), bone area mm^2 (BAR), bone perimeter mm (BPM), cortical thickness mm (CSTH) pore number, pore volume mm^{-1} and pore %.

The trabecular bone properties were measured using percentage of bone volume % (BV/TV), surface to volume ratio mm^{-1} (BS/BV), bone surface density (BS/TV) mm^{-1}, trabecular thickness mm (Tb.Th), trabecular separation mm (Tb.Sp) and trabecular number mm^{-1} (Tb.N). The trabecular bone pattern factor mm^{-1} (Tb.Pf) describes the connectivity of the trabecular bone. A lower Tb.Pf indicates that the trabeculae are better connected and a higher Tb.Pf indicates that the trabecular area has a less connected structure. The structure model index (SMI) describes the degree of plate shaped and rod shaped architecture in the trabecular bone. A structure that contains only plate shaped architecture will have an SMI of 0, and architecture that contains only rod shaped architecture will have an SMI of 3. The degree of anisotropy (DA) measures the preferential alignment of the trabecular bone; if a structure is isotropic it will have a value of 0 while anisotropic structures will have a higher number. The fractal

dimension (FD) was calculated using Kolmogorov's box counting method discussed in Chappard *et al* (2001) (9).

7.2.4. Biomechanical testing

Right tibias for the three groups OVX, GOAT OVX, and GOAT OVX ALD were biomechanically tested in an Instron texture analyser. The tibias were tested using a three point bending test developed by Stumer *et al* (2005) (8). Each tibia was placed on a purpose built base and subjected to bending using a rolling stamp. The speed of stamp was 50mm s^{-1} with a 500N load cell and data was displayed in a force-displacement graph. Three measurements were recorded and expressed as:

- Ultimate load (N). The maximum force recorded in the test.
- Displacement (mm). The amount of deformation the tibias underwent during testing.
- Extrinsic stiffness or structural strength (N/mm). The slope of the linear portion of the force deformation curve as was based on a best fit linear regression.

7.2.5. Statistical analysis

Data were analyzed in the statistical package "SYSTAT" (version 11) (Systat, Chicago, USA). μCT and biomechanical measurements were for the most part normally distributed and were, therefore, amendable to parametric analysis. Where non-normal data was found a log transformation was required to obtain near normal distribution on graphical analysis. μCT and biomechanical measurements were examined by one-way ANOVA and Tukey post-hoc testing. Significance was set at $p<0.05$.

7.3. Results

There was no significant difference in any of the cortical bone measurements between the GOAT and GOAT OVX rats (Table 7-2).

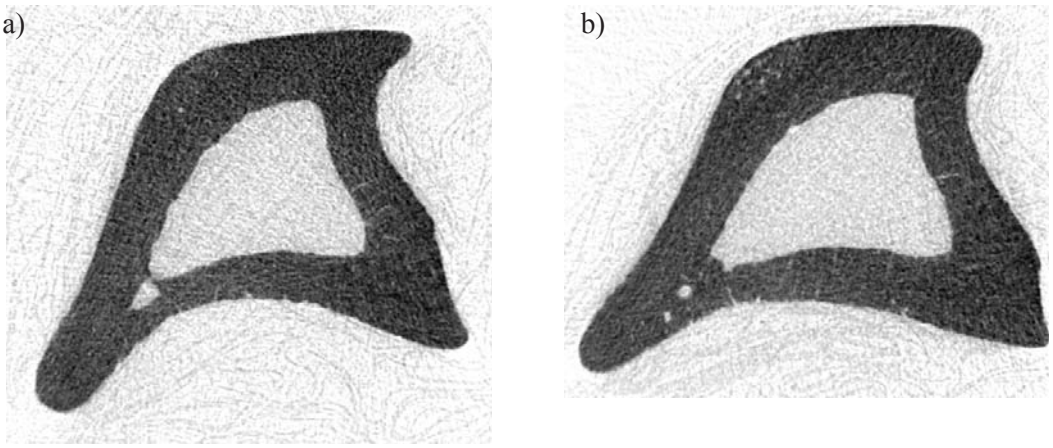

Figure 7-1. Sample µCT scans of proximal tibia cortical bone from a) a GOAT OVX rat, and b) a GOAT OVX ALD rat.

Table 7-2. Means, SD's and significant differences for µCT measurements for the cortical bone of the tibia.

Measurement	GOAT OVX	GOAT OVX ALD	P-value
BV	4.67 ± 0.31	4.70 ± 0.20	0.812
BS	35.9 ± 2.47	35.4 ± 1.50	0.614
BS/BV	7.68 ± 0.30	7.55 ± 0.32	0.370
BAR	5.82 ± 0.39	5.85 ± 0.25	0.812
BPM	25.2 ± 1.82	24.7 ± 1.20	0.466
CSTH	0.463 ± 0.025	0.474 ± 0.025	0.331
Pore number	140 ± 29.3	146 ± 54.8	0.794
Pore volume	0.001 ± 0.001	0.001 ± 0.001	0.845
Pore %	0.029 ± 0.016	0.028 ± 0.014	0.863

Means, Standard deviations (SD) and significant differences for the Tibia µCT cortical bone measurements, for the three groups. (n=10 for each group). BV= bone volume, BS= bone surface, BS/BV= bone surface to volume ratio, BAR=bone area, BPM=bone perimeter, CSTH=cortical thickness, Pore number=the number of contained spaces within a closed surface, Pore volume= the volume of contained spaces within a closed surface, Pore number= the percentage of contained spaces within a closed surface. Values with different letters are significantly different ($p < 0.05$) between diets. GOAT OVX = ovariectomized rats that received goat milk diet, goat ovx ald = ovariectomized rats that received goat milk diet and alendronate.

The connectivity of trabeculae were significantly reduced the GOAT OVX ALD rats compared to GOAT OVX rats, which was reflected in a higher Tb.Pf score ($p<0.004$). The trabecular thickness (Tb.Th) was significantly less in the GOAT OVX ALD rats

compared to the GOAT OVX rats ($p<0.006$). GOAT OVX ALD rats had a significantly higher bone surface to volume ratio (BS/BV) than GOAT OVX rats ($p<0.001$). The fractal dimensions of GOAT OVX ALD rats trabecular bone was significantly lower than GOAT OVX rats ($p<0.018$), suggesting that the GOAT OVX ALD rats had less complex trabecular surfaces. The GOAT OVX rats had significantly lower SMI results than the GOAT OVX ALD rats ($p<0.048$), suggesting that the GOAT OVX rats had a higher prevalence of plate shaped trabeculae.

Table 7-3. Means, SD's and significant differences for µCT measurements for the trabecular bone of the tibia.

Measurement	GOAT OVX	GOAT OVX ALD	P-value
BV/TV	11.6 ± 3.28	9.91 ± 3.02	0.101
BS/BV	49.9 ± 4.2[a]	55.3 ± 4.9[b]	**0.001**
BS/TV	5.70 ± 1.14	5.48 ± 1.25	0.509
Tb.Th	0.078 ± 0.006[a]	0.072 ± 0.005[b]	**0.006**
Tb.Sp	0.392 ± 0.069	0.421 ± 0.120	0.606
Tb.N	1.49 ± 0.31	1.37 ± 0.36	0.399
Tb.Pf	16.1 ± 2.82[a]	19.2 ± 3.05[b]	**0.004**
SMI	2.01 ± 0.18[a]	2.16 ± 0.18[b]	**0.048**
DA	1.97 ± 0.11	2.02 ± 0.11	0.337
FD	2.09 ± 0.02[a]	2.07 ± 0.03[b]	**0.018**

Means, Standard deviations (SD) and significant differences for the Tibia µCT trabecular bone measurements, for the three groups. (n=10 for each group). BV/TV=percentage of bone volume, BS/BV=bone surface to volume ratio, BS/TV=bone surface density, Tb.Th=trabecular thickness, Tb.Sp=trabecular separation, Tb.N=trabecular number, Tb.Pf=trabecular bone pattern factor, SMI=structure model index, DA=the degree of anisotropy, and FD= the fractal dimension. Values with different letters are significantly different ($p< 0.05$) between diets. GOAT OVX = ovariectomized rats that received goat milk diet, goat ovx ald = ovariectomized rats that received goat milk diet and alendronate.

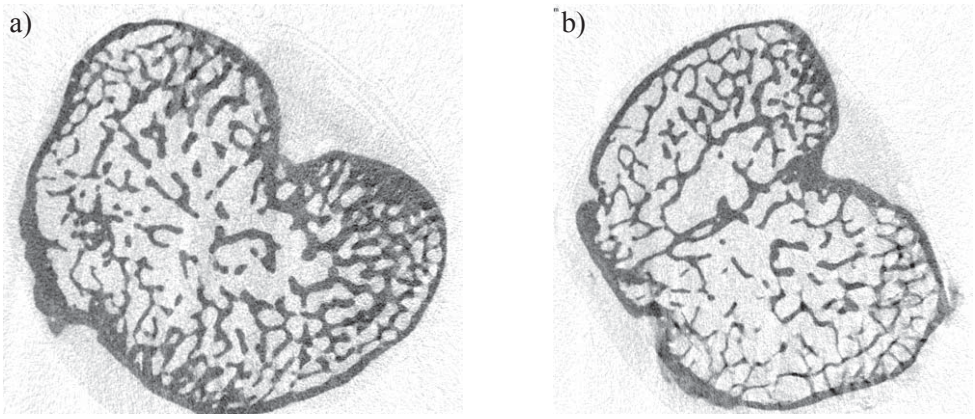

Figure 7-2. Sample µCT scans of proximal tibia trabecular bone from a) a GOAT OVX rat, and b) a GOAT OVX ALD rat.

There was no significant difference between the GOAT OVX and GOAT OVX ALD rats in the biomechanical testing (Table 7-4).

Table 7-4. Means, SD's and significant differences for biomechanical testing extrinsic measurements of the proximal tibia.

Measurement	GOAT OVX	GOAT OVX ALD	P-value
Ultimate load (N)	145 ± 20.0	138 ± 26.9	0.555
Displacement (mm)	1.405 ± 0.22	1.37 ± 0.269	0.756
Stiffness (N/mm)	105 ± 17.0	105 ± 29.0	0.967

Means, Standard deviations (SD) and significant differences for the Tibia biomechanical measurements, for the three groups. Values with different letters are significantly different ($p < 0.05$) between diets. GOAT OVX = ovariectomized rats that received goat milk diet (n=13), GOAT OVX ALD = ovariectomized rats that received goat milk diet and alendronate diet (n=13).

7.4. Discussion

Trabecular bone is more sensitive to the effects of osteoporosis than cortical bone in both humans and rats. In the case of women and rats it has been shown that osteoporosis causes the determination of the trabecular network and compromises the strength of bone under loading (10-13). Due to its largely trabecular content the proximal tibia is ideal in assessing the biomechanical competence of osteoporotic bone (8). The strength of a trabecular structure has been associated with the connectivity,

orientation and quantity of bone within that structure. Kleerekoper *et* al (1985) concluded that the trabecular structure was as important as bone mass in determining the biomechanical competence of bone in post-menopausal women, and fracture was more likely to occur in areas with less connected trabeculae (14). In 1992 Hahn *et al* (1992) developed a new method of quantifying the connectedness of the different shaped surfaces within the 3-dimentional structure of trabecular bone known as 'Trabecular bone pattern factor' (Tb.Pf) (15). This method described the surfaces as either 'concave' or 'convex', and was related to the number of plates and rod shaped trabeculae that made up the trabecular architecture (15). A biomechanically compromised trabecular structure has greater quantities of convex surfaces representing isolated, and therefore, disconnected struts within the micro-architecture (15). This type of arrangement is found in situations where bone loss has occurred, such as osteoporosis resulting in weakened bone tissue (16, 17). In the current trial the GOAT OVX ALD rats displayed higher Tb.Pf values indicate greater convexity (convex surfaces) or disconnected struts within the structure. This suggests that the dosing the GOAT OVX rats with alendronate lead to a weakening of the trabecular region. This was, however, not reflected in the biomechanical testing done on the proximal tibia. Another measure of connectivity is 'Fractal dimension' (FD) which measures the complexity of the surface of a structure (6), or in the case of trabecular bone, how the bone fills the gaps within the trabecular structure. A widely spaced trabecular arrangement will be less biomechanically sound than if the same amount of bone is arranged with more connections, even if the more complex arrangement means the struts are thinner (14). The more connections a strut has means that there is less area along the strut that is unsupported, thereby increasing the amount of load the structure can carry before failure (18, 19). In the case of this trial the GOAT OVX rats had a significantly higher degree of FD (more significant connectivity) compared to the GOAT OVX ALD rats.

Earlier studies have examined the effect of ovariectomy on the shape of trabeculae. Historically it has been found that ovariectomy changes the shape of the trabeculae from a largely plate-shaped configuration to a rod-like shape (20). This change in structure was observed by Laib *et al* (2001) within the first week after ovariectomy in female rats ovariectomized at 6 months of age (21). The SMI results of the GOAT OVX rats and GOAT OVX ALD rats were both lower than the ovariectomized rats in Laib *et al* (2001) study (2.69 ± 0.20) (21), suggesting that the goat milk diet may help protect the

trabeculae from the effects of ovariectomy. The current trial also showed a significantly higher prevalence of plate-shaped trabeculae in the GOAT OVX rats compared to the GOAT OVX ALD rats. This result implies that goat milk alone helps protect the trabeculae from the full effects of bone loss, and structural changes known to occur in ovariectomized rats, whereas the co-administration of goat milk and alendronate are not as effective. The findings in the current trial do not match with the work done by Hu *et al* (2002), who found that dosing mature female dogs with alendronate for 12 weeks reduced the shift from the plate shaped trabeculae within the vertebrae of the lumbar spine and humerus (22). An ovariectomized rat trail also found that the risedronate (another Bisphosphonate drug) was able to maintain the plate shaped trabeculae of the proximal tibia at both high and low dose rats (0.5 & 2.5 mg/kg) (23). It is uncertain as to why goat milk and alendronate had a differing effect.

Ovariectomy has been associated with the increase the thickness of trabeculae, probably as a means of protecting the structure from the increased porosity due to bone loss (24). The thicker struts are formed when the existing trabeculae merged and rearranged their structure (10). The addition of alendronate as a drug therapy has been shown to further thicken the trabecular struts in rats nonhuman primates, and humans (25-27). However, the results of the current trial are contrary to this, where the addition of alendronate did not cause significantly greater trabecular thickness compared to the rats not dosed with the drug. BS/BV measures the surface to bone volume ratio which also provides a general indication of trabecular thickness. In previous work investigating the effects of Bisphosphonates on osteoporotic bone in aging women, research has found that not only did the drug 'Risedronate' decrease the shift from plate shaped trabecular to rod shaped structures, but they also had lower BS/BV measurements (28). This is contrary to what was found in this trial where the GOAT OVX ALD rats had a significantly greater surface to bone volume ratio than the GOAT OVX rats. In contrast, a significant increase in BS/BV was seen in the humerus and lumbar spine in mature female dogs dosed daily with alendronate (0.5/mg/kg) for 12 weeks compared to the placebo group (22). So in the current trial when ovariectomized rats were fed goat milk, the inclusion of alendronate had a detrimental effect on the micro-architecture of trabecular bone. However, when the tibias were subjected to three-point bending no significant differences where found between the two groups fed goat milk. While the addition of alendronate to a goat milk diet may have caused changes in the micro-architecture of the

trabecular bone, it may not have been enough to significantly impact the actual bone strength when subjected to bending. Another possibility may be that this testing method introduced an effect that masked the weakened trabecular bone in the GOAT OVX ALD group. The testing method used in the current study used bone specimens that contained both cortical and trabecular bone. It has been suggested that biomechanical testing of bone specimens that contain both cortical and trabecular bone reduces the sensitivity of the test to detect changes in the trabecular bone (7).

7.5. Conclusion

This study found that dosing ovariectomized rats fed a goat milk diet with alendronate resulted in a loss of connectivity between trabeculae struts, increased the prevalence of rod shaped trabeculae and a greater surface to volume ratio compared to those rats not dosed with alendronate. This suggests that dosing the GOAT OVX rats with alendronate weakened the structure of the trabecular region of the proximal tibia. However this was not reflected in the biomechanical testing of the same region. The current trial also showed that the GOAT OVX rats had significantly greater trabecular thickness compared to the GOAT OVX ALD rats.

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8. Discussion and Conclusion

Several animal studies have indicated that goat milk has a beneficial effect in mineral accretion and retention in bone. These studies all indicated that goat milk not only surpassed the non-milk diets, but also the cow's milk diets in mineral absorption and retention (1-4). To date no human studies and only one animal study have investigated the effects of goat milk as a dietary supplement in assisting with the prevention of age-related bone loss. No work has been done to assess the effect of a goat milk diet on architectural changes of cortical bone in growing or ovariectomized rats, or the structure of trabecular bone in ovariectomized rats. More research is required in order to understand the benefits of goat milk on bone health to possibly improve growth or protect against age-related bone loss.

During periods of rapid bone growth, expansion occurs at both the endosteal and periosteal surfaces of long bones. Studies investigating the effects of cow's milk supplements in the diets of children and adolescents have reported increased expansion at both endosteal and periosteal surfaces of the mid-radius and mid-second metacarpal bones (5, 6). In Chapter 4 the increased mineral retention in the growing rats fed the milk diets resulted in significantly smaller marrow cavities at the mid and proximal section of the femoral shaft. The increased apposition at the endosteal surface did not result in an enlarged bone area, nor was there any significant expansion at the periosteal surface. Despite this lack of a significant difference in bone area there was significantly greater mineral composition in the rats fed the milk diets, indicating a higher degree of mineralization in the femur. Human studies in children have shown similar results, where the addition of milk into their daily diet resulted in increased BMD and BMC (7-9). Over an 18 month period adolescent girls given 568ml of milk a day increased their BMD by 9.6% and BMC by 27% compared to 8.5% and 24.1% respectively in girls who maintained their habitual diet (8). Milk contains bioactive components that have demonstrated a physiological effect that increases mineral uptake and retention in bone (10). The three formulated diets used in Chapter 4 had a similar composition of minerals, protein and fat content. However the rats fed the non-milk diet had less mineral retention compared to the rats fed the milk diets. It is therefore possible that the bioactive components of the milk diets aided in the bioavailability of essential minerals such as calcium for absorption into the body (11, 12).

In Chapter 2 altering the levels of the bioactive component CPP (by manipulating the ratio of whey and casein) affected the degree of calcium uptake during the period of rapid bone growth in young rats, where the higher levels of CPP (casein:whey ratio of 57:43 and 80:20) had greater mineral absorption compared to a lower level (casein:whey ratio of 20:80). The study in Chapter 2 also found that the fats in the diets may have had an effect on the absorption of calcium; although neither the vegetable oil mix or goat milk fats resulted in increased mineral retention by the end of the study. The rats fed the diet with a ratio of 87:17 whey:casein (Diet 6) with the lowest levels of goat milk fat, had the lowest calcium absorption. Another study reported a similar result where both growing and ovariectomized rats fed diets which used vegetable oil and goat skim milk powder as their fat source, had less calcium absorption than rats fed a formulated whole goat milk diet (1). Kruger *et al* and other researchers have suggested that the composition of goat milk fat aided digestibility and mineral absorption into the body (1, 13, 14). Several studies have recommended dairy as the primary source of dietary calcium compared to calcium from $CaCO_3$ (10, 15). In growing female rats, diets formulated from milk products improved bone material, macro and micro-structure compared to a non-milk diet that uses calcium carbonate and egg protein (10). The rats fed the milk formulated diet had greater calcium content, density, cortical thickness, and area in the femur than rats fed a non-milk diet with $CaCO_3$ (10). Healthy postmenopausal women given a milk supplement showed less bone remodelling suppression compared to the women given the $CaCO_3$ supplement (15). The authors suggested that the less aggressive suppression of remodelling was preferable for skeletal health (15).

Maximising 'Peak bone mass' prior to the onset of post-menopausal can reduce the rate of bone loss in old age (16). The ovariectomized rats fed the goat and cow's milk diets from weanlings in Chapter 5 had reduced bone loss from the endosteal surface of the femoral shaft compared to the rats fed the non-milk diet. However, the presence of the vitamin A deficiency made it difficult to draw conclusions from these data. To address this within the scope of this thesis, general comparisons have been drawn between the SHAM and OVX rats discussed in Chapter 3 and the SHAM and OVX rats fed the two milk diets in Chapter 5. These comparisons show that the SHAM and OVX rats discussed in Chapter 5 had smaller bone areas in the femoral shaft due to less expansion

at the periosteal surface as seen by smaller total cross sectional area (overall bone size) and increased marrow cavity areas compared to the rats in Chapter 3. Therefore, this reduced bone area may be reflecting one of the consequences of the vitamin A deficiency.

Chapter 3 showed that ovariectomy altered the size and shape of the femur by comparing the cross sectional areas of contiguous slices along the shaft of the femur. The overall findings of periosteal and endosteal expansion in ovariectomized rats are in agreement with previous research (17-19). However, the expansion of the proximal regional of the femoral shaft compared to the distal region suggests that the rate of endosteal resorption in ovariectomized rats is uneven. This regional variation was found again in all ovariectomized rats including those fed the goat and cow's milk diets in Chapter 5. However, when compared to the SHAM rat there was no significant difference in overall marrow cavity size. No papers could be found that discussed this regional variation, as most studies tend do one or two cross sectional measurements but are region specific: the interest is therefore focused on either the distal region or the mid-shaft, not both. A comparison between data from the GOAT OVX and GOAT OVX ALD rats of Chapter 5 and the SHAM rats from Chapter 3 also confirmed that the marrow cavities of the rats fed the milk diets were less affected by ovariectomy surgery. Therefore the long term consumption of the goat milk and cow's milk diet was beneficial in helping to prevent bone loss from the endosteal surface.

In Chapter 5 it was found that the GOAT OVX rats had a tendency towards having larger bone areas in the femoral shaft compared to the GOAT OVX ALD rats ($p<0.063$), although, there was no significant differences found between the GOAT OVX rats and GOAT OVX ALD rats in BMC or BMD. Therefore, the smaller bone area of the GOAT OVX ALD rats may have been due to the reported effects of alendronate on the periosteal envelope, where the drug was found to suppress expansion (20). The toughness testing in the proximal region of the shaft found that the ovariectomized rats fed the GOAT OVX ALD rats required greater amounts of energy (J/mm^2) to fracture than the GOAT OVX rats given the placebo. Further investigation was unable to determine whether the size of the sample (cross sectional area) or the bone area significantly influenced the amount of impact energy (J) needed to fracture the proximal segment. Therefore it is uncertain if alendronate aided in increased

mineralisation of the proximal segment. If this was the case then the results found in Chapter 6 would be in contrast to past research were increased mineralisation was reported to reduce the ability of the bone structure to absorb energy in bone samples taken from elderly human cadavers (21). Bone mineralisation from alendronate use in dogs at a dose rate of 1mg/kg/day also showed reduced toughness compared to dogs given a placebo, suggesting the increased mineralisation from alendronate was compromising the toughness of the bone material (22). Further research is needed to explore what caused the difference in toughness of the bone material between the ovariectomized rats fed the goat or cow's milk diet and those rats fed the milk diets and dosed with alendronate.

The results of Chapter 4 and 5 both suggest that there were only slight differences between the rats fed the cow's milk diet compared to the rats fed the goat's milk diet in mineral retention in the femur as both growing rats and post ovariectomy. While this did not result in overall differences in bone area measurements, a closer comparison within each region in Chapter 6 showed that the COW OVX ALD rats had larger bone areas and bone sizes in the mid-shaft compared to the GOAT OVX ALD rats. Further investigation found that the amount of impact energy (J) needed to break the mid-shaft of the GOAT OVX ALD rats increased relative to the increase in bone area. Therefore if the COW OVX ALD rats had the same area of bone it would have taken less impact energy (J) to fracture the mid-shaft (Segment B). This suggests that the COW OVX ALD rats laid down greater quantities of weaker cortical bone and the co-administration of goat milk and alendronate resulted in smaller tougher bone. Previous work has suggested that fracture toughness K_c and G_c are influenced by the density of a material rather than that materials thickness (23, 24). This could support the findings here where the smaller areas of the GOAT OVX ALD rats had a tendency to be able to absorb greater amounts of impact energy before fracturing compared to the mid-shaft bone segment of the COW OVX ALD rats. However, further investigation is needed to determine the mechanisms that caused this regional difference in bone between rats fed milk diets.

Regional variations in biomechanical behaviour within a treatment were found in both Chapter 3 and Chapter 6. Regional variations in the biomechanical properties of bone have been reported before, where the neck of the femur has been shown to be tougher

under both tensile and shear loading compared to the tibia shaft (25). The SHAM and OVX rats in Chapter 3 demonstrated regional variations in the maximum amount of axial load (N) that the segment could be compressed under before fracture, although there was only a tendency for that variation to be as a result of ovariectomy surgery ($p<0.077$). In Chapter 6 the co-administration of cow's milk with alendronate resulted in an uneven effect on the material toughness across the proximal and mid-shaft of the femur, whereas the GOAT OVX ALD rats had a more even distribution on material toughness.

Trabecular bone is more sensitive to ovariectomy with between 20-30% bone lost compared to 5-10% in cortical bone (26), and its strength is measured by the orientation and quality of the bone as well as the connectedness of the individual trabeculae (27). The effect of bone loss is similar in women and rats; the trabeculae become thinner and eventually entire trabeculae are removed resulting in a more porous structure (28, 29). The remaining trabecular struts thicken which in turn change the shape of the trabeculae from a plate shape to a more rod like shape (28, 29). The effect of ovariectomy on trabecular bone was examined by DEXA in the lumbar spine and μCT in the proximal tibia. No significant differences were found in the lumbar spine area, BMC or BMD between GOAT OVX rats and GOAT OVX ALD rats in Chapter 5. However, examination of the micro-architecture of the trabecular region of the proximal tibia in Chapter 7 revealed that GOAT OVX ALD rats had increased prevalence of rod shaped trabeculae, increased surface to bone volume ratio, reduced connectivity between trabeculae struts, and thinned trabeculae compared to the GOAT OVX rats. The increase in surface to bone volume ratio has been reported in mature female dogs, however, the authors also reported that dogs dosed with alendronate displayed a decrease in the shift to rod shaped trabeculae (30). This is in contrast with the findings in the GOAT OVX ALD rats discussed in Chapter 7, where there was a greater prevalence for rod shaped trabecular compared to the GOAT OVX rats. It is uncertain why the ovariectomized rats fed goat milk and dosed with alendronate didn't decrease the architectural shift of plate shape trabeculae to rod shaped trabeculae, as seen in other research, and further work is required to investigate this finding.

The findings reported in this thesis suggest that a goat milk formulated diet improves the absorption of minerals such as calcium during periods of rapid growth, and reduces

the effects of bone loss in ovariectomy induced post-menopausal osteoporosis. However, the vitamin A deficiency in what was supposed to be the diet control group, and sham ovariectomy surgery group, make it difficult to draw significant conclusions. The inclusion of the cow's milk diet in the trials, allowed for similarities to be drawn between goat's milk and cow's milk as a dietary supplement.

In terms of the possible implications for humans, this research suggests that goat milk is a reasonable substitute for cow's milk, in supplying dietary calcium throughout life from childhood to old age. Comparing the co-administration of alendronate with goat milk indicated an effect on cortical and trabecular bone morphology, and on the toughness of the bone material within the femoral shaft. Goat's milk should be examined in humans as a dietary supplement to increase the quality and quantity of bone material.

8.1. Conclusion

In conclusion, the long term consumption of both goat milk and cow's milk formulated diets assisted in reducing the effects of bone loss brought on by ovariectomy in mature female rats.

However further work is needed to investigate the co-administration of the milk diets with alendronate on fracture toughness, mineralisation and micro-architecture in both cortical and trabecular bone. The co-administration of either goat or cow's formulated diets with alendronate increased the toughness of the bone material (energy absorbed (J/mm^2)) in the proximal femoral shaft compared to rats fed the milk diets and given the placebo. In the mid-shaft the GOAT OVX ALD rats had a tendency to have tougher bone material than the COW OVX ALD rats. However, μCT results suggest that the administration of alendronate to a goat milk diet may weaken the trabecular structure of the proximal tibia.

8.2.Recommendations for Future Research

- Aspects of this study would have to be re-examined to compare differences between rats fed a both milk diets and rats fed a healthy control due to the

vitamin A deficiency in the non-milk diet of both growing and mature ovariectomized rats. In particular the effect of the diet and drug combinations on trabecular bone should be measured using μCT.

- The effect of sodium alendronate on collagen should be investigated to determine the role of collagen on the toughness of cortical bone between of both goat and cow's formulated diets with and without alendronate.

- The potentially weaker mineralisation of cortical bone from rats fed a cow's milk diet versus a goat milk diet should be investigated, particularly with the co-administration of alendronate.

- Further pre-clinical research should be done using an animal that more closely matches human cortical bone and biomechanical testing on micro-crack propagation between goat milk and goat milk + sodium alendronate should be done.

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