THE EFFECT OF LONG-TERM EXERCISE AND DIET RESTRICTION ON BONE GEOMETRY AND BONE STRUCTURE IN RATS

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submitted by
Marko Bodnar
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Supervisor:
Univ. Prof. Dr. med. Dr. med. vet. Reinhold ERBEN
Institute of Pathophysiology
Department for Biomedical Sciences

Reviewer:
Ao. Univ. Prof. Dr. Monika EGERBACHER
Institute of Anatomy, Histology and Embryology
Department for Pathobiology
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Abbreviations

ANOVA Analysis of Variance
BMD Bone Mineral Density
BMC Bone Mineral Content
vBMD Volumetric BMD
BMU Basic multicellular unit
BUA Broadband ultrasound attenuation
CtTh Cortical Thickness
CtAr Cortical area
CtsubAr Cortical-subcortical area
Ctsub BMD Cortical-subcortical BMD
tCSA Total Cross sectional area
CV Coefficient of variation
DXA Dual energy X-ray absorptiometry
FM Fat mass
IDSC International DXA Standardization Committee
LBM Lean Body mass
MRI Magnetic resonance imaging
MAr Bone marrow area
OC Osteocalcin
PBM Peak bone mass
pDXA Peripheral DXA
pQCT Peripheral quantitative computed tomography
QCT Quantitative computed tomography
QUS Quantitative ultrasound
sBMD Standardized BMD
SD Standard deviation
SEM Standard error of the mean
SOS Speed of sound
WHO World Health Organization
1. INTRODUCTION

1.1. Osteoporosis

1.1.1. Definition and classification

Osteoporosis, a chronic metabolic disease of complex etiology, is defined as a progressive systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (CONSENSUS DEVELOPMENT CONFERENCE, 1993). It was a relatively rare disorder, until recent times when increased longevity and changes in lifestyle produced a large elderly population with fragile skeletons. Nowadays, with more than 200 million persons who suffer from it worldwide, osteoporosis is recognized as a major health problem that has a prevalence that is similar to that of other major diseases, such as cancer and cardiovascular disease (JOHNELL a. KANIS, 2005; JOHNELL a. KANIS, 2006).

Osteoporosis can be regarded as a form of bone atrophy. It is a condition that affects the entire skeleton. The ratio of bone mineral to collagen remains constant in osteoporosis which differentiates it from osteomalacia, a disease characterized by a relative decrease of mineral to organic material content.

Osteoporosis can occur as a primary disorder or secondarily due to some other factor.

Primary osteoporosis, which has been differently called postmenopausal, involutional, senile or idiopathic, is the most frequent metabolic disorder of the skeleton (RAISZ, 1997). Primary osteoporosis involves multiple pathogenetic mechanisms, many of which have not yet been adequately defined. With respect to changes in regional BMD pattern of fractures, hormonal changes and etiology it has been divided into type I, or postmenopausal osteoporosis, and type II, or senile osteoporosis.

Postmenopausal or type I osteoporosis accounts for the significantly greater risk for osteoporosis in women than in men generally. This syndrome develops in women during the first 15-20 years after menopause and is characterized by an excessive and disproportionate loss of trabecular bone over cortical bone leading to vertebral and appendicular bone fractures. The unique theory (RIGGS a. MELTON, 1983) supposed that type I osteoporosis developed from estrogen deficiency along with some additional factor, which is functioning
only in the presence of estrogen deficiency, and intensifies the degree and duration of the rapid postmenopausal phase of bone loss.

According to previous reports (MANOLAGAS a. JILKA 1995; KAVAGUCHI et al. 1995), cytokines, such as interleukin-1, interleukin-6, tumor necrosis factor, and prostaglandin E2, may act as paracrine mediators of estrogen action in bone. Conceivably, a genetically determined increased responsiveness of these cytokines may, in the presence of estrogen deficiency co-stimulate and incline some postmenopausal women to extreme cancellous bone loss and thus type I osteoporosis.

Type II osteoporosis (senile osteoporosis) in women and involutional osteoporosis in men happens after the age of 70 and affects women twice as frequently as men. It relates to the slow phase of age-related bone loss and results from the combined alterations in bone- and hormone metabolism and dietary factors. Overall, age-related osteopenia appears to result from inversely related changes in the pool size of hematopoietic osteoclast precursor cells and bone forming cells. The impaired balance between bone resorption and formation is usually associated with lower exposure to sunlight and reduced capacity of skin to produce vitamin D₃, insufficient dietary intake along with decreased ability of the kidney to produce 1,25(OH)₂D₃. The vitamin D₃ deficiency results in decreased calcium absorption, which consequently increases the parathyroid hormone level and often leads to secondary hyperparathyroidism (DOCHEVA et al., 2007; DUQUE a. TROEN, 2008).

This regression occurs in the entire population of aging women and men to some extent, and induces analogous losses of both trabecular and compact bone. Most of the fractures that correspond to this syndrome are those of the proximal femur and compression fractures of the vertebrae, proximal humerus and tibia although various other fractures occur also at sites with mixtures of cancellous and cortical bone (RIGGS a. MELTON, 1986).

Nevertheless, some reports have suggested that estrogen deficiency is important for the pathogenesis of both types of osteoporosis and in both men and women (RIGGS et al., 1998).

Secondary osteoporosis occurs equally in men and women at any age, and it accounts for less then 5% of all cases. This type of osteoporosis is caused by another underlying disease,
deficiency or drug. It may arise as a consequence of various lifestyle factors (eating disorders, smoking, alcoholism), disease processes (endocrinopathies, gastrointestinal tract disease, hepatobiliary disease), and treatment regimens that comprise corticosteroids or chemotherapeutic agents.

In fact, there are a lot of different causes that can induce osteoporosis (TEMPLETON, 2005):

a) Lifestyle factors:  Anorexia nervosa  
Excessive protein intake  
Smoking  
Excessive alcohol intake

b) Endocrinopathies:  Hyperthyroidism  
Hyperparathyroidism  
Cushing’s syndrome  
Type 1 diabetes mellitus  
Hypogonadism

c) Systemic diseases:  Gaucher’s disease  
Mastocytosis  
Rheumatoid arthritis  
Ankylosing spondylitis  
Psoriasis

d) Organ dysfunction:  Cystic fibrosis  
Asthma  
Chronic obstructive pulmonary disease  
Renal failure  
Primary biliary cirrhosis  
Inflammatory bowel disease  
Celiac sprue  
Organ transplantation
e) Medications:  
   Glucocorticoids  
   Diuretics  
   Antiepileptics  
   Methotrexate  
   Cyclosporin A  
   Excess thyroid hormone replacement  
   Alkylating chemotherapeutic agents  
   Gonadotropin-releasing hormone agonist  

f) Neoplastic conditions:  
   Multiple myeloma

1.1.2. Pathophysiology

1.1.2.1. Basic principles

Osteoporosis is a heterogeneous, multifactorial disease that depends on both environmental and hereditary factors. Factors such as genetics, estimated to be accountable for about 50-70% of the variance in bone mass (SLEMENDA et al., 1991; JOUANNY et al., 1995; EISMAN, 1999), are innate and can not be influenced. The other factors, such as nutritional intake, physical activity, and body mass, can be modified, thereby decreasing the risk of osteoporosis and its consequences (CUMMINGS et al., 1995). Furthermore, a good comprehension of the physiology of bone turnover and pathophysiology of osteoporosis can provide the basis for better understanding of fracture risk, of the observed alterations in bone turnover markers, and of the action of pharmacologic agents for the deterrence and treatment of osteoporosis.

According to a large amount of information based on laboratory studies, there are three critical factors that can lead to fragile skeleton (RAISZ, 2005):

(1) Inability to attain optimal peak bone mass and strength during growth. Although it is largely determined by genetic background (ALBAGHA a. RALSTON, 2006; RALSTON, 2007), nutrition and life-style can have an effect during growth and development. Additionally, sufficient calcium and vitamin D intake and appropriate physical activity may not only augment peak bone mass but also slow bone loss and decrease fracture risk throughout life (LOCK et al., 2006).
(2) Exaggerated bone resorption resulting in diminished bone mass and microarchitectural deterioration of skeleton. This seems to be less dependent on hereditary background (POCOCK et al., 1987; MICHAELSSON et al., 2005). Estrogen deficiency, in postmenopausal women, but also in older males, plays a pivotal role in intensifying bone loss (FALAHATI-NINI et al., 2000; AMIN et al., 2006). Calcium and vitamin D insufficiency, which may lead to secondary hyperparathyroidism, are also important (LIPS, 2001).

(3) Defective formation response to increased bone resorption during remodeling. A gradual decline in the ability to create sufficient amounts of new bone to retain bone mass during remodeling may begin shortly after peak bone mass has been reached (LIPS et al., 1978). In order to elucidate the underlying mechanisms, it is indispensable to understand the changes in local and systemic growth factor production (CHARATCHAROENWITTHAYA et al., 2007; ZHOU et al., 2006). Moreover, the involvement of cytokines in both accelerated resorption and in impaired formation has clearly opened new avenues in our understanding of the physiopathological aspects involved.

1.1.2.2. Age related bone loss
Age-related bone loss is characterized as one of the most important factors in osteoporosis and is the result of an imbalance in the volumes of bone resorbed and formed in each focal basic multicellular unit (BMU) that remodels the bone tissue. Osteoporosis in elderly subjects (“senile osteoporosis”), has also been attributed to endocrine alterations, like sex hormone deficiencies, secondary hyperparathyroidism (RIGGS et al., 1998). However, the specific relations between these and the changes of local bone remodeling processes with age are not known. Along with the loss of bone mass, senescent bone tissue is characterized by several cellular, biochemical and physical differences, such as microdamage accumulation and bone structure deterioration, collagen cross-linking alterations or changes in cellular numbers, secretion of growth factors and other activities that contribute to the age dependent increase in bone fragility during the normal aging process (NICOLAS et al., 1994; CHAN a. DUQUE, 2002; NYMAN et al., 2007; CAO et al., 2003; SEEMAN, 2008).
1.1.3. Diagnosis

Osteoporosis is a silent disease and fractures often develop before the diagnosis of the disease is made. The Diagnosis of osteoporosis is generally based on measurement of bone mineral density (BMD) thereby defining thresholds - a cutoff for BMD, which encompasses most patients with osteoporotic fractures. This procedure is feasible because of the Gaussian distribution of bone density values, where bone density is expressed in relation to a reference population in terms of standard deviation (SD) units (KANIS, 2002). When SD units are used in relation to a control population of gender matched, young, healthy adults at peak bone mass, the measurement is defined as the T score. The Z score represents the standard deviation compared with the mean BMD of an age and gender matched control population.

The recommended site for diagnosis is the proximal femur but the lumbar spine can be also used. The World Health Organization (WHO) and the International Osteoporosis Foundation have established four diagnostic categories for women based on dual energy X-ray absorptiometry (DXA) measurements (KANIS a. GLÜER, 2000; ASSESSMENT OF FRACTURE RISK AND ITS APPLICATION TO SCREENING FOR POSTMENOPAUSAL OSTEOPOROSIS., 1994).

- Normal Bone Density: hip BMD is greater than 1 SD below the young adult female reference mean (T score ≥ -1).
- Low bone mass (osteopenia): hip BMD more than 1 SD below the young adult female mean, but less than 2.5 SD below this value (T score < -1 and > -2.5).
- Osteoporosis: hip BMD 2.5 SD or more below the young adult female mean (T score ≤ -2.5).
- Severe osteoporosis (established osteoporosis): hip BMD 2.5 SD or more below the young adult average in the presence of one or more fragility fractures.

The WHO standards for the densitometric diagnosis of osteoporosis strictly apply only to white postmenopausal women. Unfortunately, convenient diagnostic threshold values for men are less well defined than for women. At present, no agreement has been established about which T score to determine the densitometric diagnosis of osteoporosis in men.
However, the few existing studies (DE LAET et al. 1998; KANIS et al., 2001) show that the risk of hip fracture is analogous in men and women for any given BMD. Such reports specify that a similar cutoff value for hip BMD that is used in women can be used in the diagnosis of osteoporosis in men that is, a value for BMD 2.5 SDs or more below the average for women (KANIS a. GLÜER, 2000). Similarly to that, men with T scores that are 2 to 2.5 standard deviations below the reference mean are at significantly increased risk of fracture and should most likely be given treatment (ORWOLL, 2000).

1.1.4. Male osteoporosis

Although women have been the main focus of osteoporosis research, it is increasingly evident that osteoporosis in elderly men is a frequent and severe condition (KELLIE a. BRODY, 1990; LEGRAND et al. 2000). About one quarter of all osteoporotic hip fractures occur in men (ORWOLL a. KLEIN, 1995). In men, bone loss and consequent development of osteoporosis is either secondary to other diseases or medications or because of aging. Nevertheless, in 40% to 60% of all occurrences no cause for the disease can be found, and this is termed primary or idiopathic osteoporosis (SEEMAN et al., 1983; TORTORA, 2008).

Male osteoporosis manifests differently from osteoporosis in women. Men achieve approximately 10% greater peak bone mass than women therefore osteoporosis tends to develop about a decade later. Testosterone and estradiol levels decrease slightly with aging of men.

1.1.4.1. Senile osteoporosis

Age-related or involutional osteoporosis in men is related to both cortical and trabecular bone loss. Histomorphometric studies have demonstrated that bone loss with aging results from a prevalent decline in bone formation and osteoblastic activity with a sustained osteoclastic function (CLARKE et al., 1996). According to a recent report, bone resorption biomarkers increase significantly in men, after the age of 60 years, while indicators of bone formation remain stable (SZULC et al., 2001). This age-related inequity in bone turnover results in a net loss of bone volume which consequently leads to the occurrence of osteoporosis.

Senile osteoporosis in both men and women can result from imbalance in the demands for and intake of calcium and vitamin D. Moreover, the level of parathyroid hormone has been shown
to increase in with age, partly as a result of low dietary calcium intake, which is not compensated for by an increase in 1,25-dihydroxy vitamin D production (KANIS, 1994).

Aging in men is also attributed to changes in reduction of gonadal hormone levels and growth factors that affect bone metabolism and strength (NICOLAS et al., 1994; CENTER et al., 1999; GRAY et al., 1991). As testosterone and estradiol are related to male bone metabolism, the age-related alterations could conduce to the development of senile osteoporosis in men.

1.1.4.2. Secondary osteoporosis

The most ordinary causes of secondary osteoporosis in men include hypogonadism, glucocorticoid therapy, gastrointestinal disease and alcohol abuse (FRANCIS et al., 1989; KELEPOURIS, et al., 1995).

1.1.4.2.1. Hypogonadism

Hypogonadism in men is related to low bone mineral density (BMD) and osteoporosis (GREENSPAN et al., 1986). This decline in BMD is usually connected with a higher risk for fractures, both of the spine and the hip (ORWOLL, 1996). Treatment with gonadotropin-releasing hormone agonists generates hypogonadism in aged men with subsequent reductions of serum testosterone levels. During the first year of treatment, this results in a high-turnover bone-loss state, resulting in diminished vertebral bone density (GOLDRAY et al., 1993). Some of the studies have demonstrated that testosterone replacement in hypogonadal adult men is related to improvements in BMD (ORWOLL, 1996). However, the cellular response to testosterone withdrawal seems to be complex in many ways. Gonadal insufficiency in men in early stages is characterized by rapid bone loss and augmented remodeling. This early period of bone loss in men is later followed by a phase of lower remodeling rates, coinciding with a relative decrease in bone formation. Thus, trabecular bone mass declines in both genders as a result of hypogonadism, nevertheless, the structural nature of bone loss differs qualitatively, since perforation of cancellous bone plates is more frequent in women (SEEMAN, 1995).

1.1.4.2.2. Glucocorticoid therapy

Long-term exogenous glucocorticoid therapy causes bone loss and accounts for approximately one in six cases of male osteoporosis (SEEMAN et al., 1983). The extent of bone loss is related to the duration of therapy and the dosage of the steroid.
The bone loss is most evident in the first 12 months of medication and develops more rapidly in cancellous than compact bone. In patients who were exposed to glucocorticoid therapy for 5 years, up to 20% diminution of spinal trabecular bone has been observed (REID et al. 1997). The main mechanism of glucocorticoid-induced bone loss includes alterations in the balance between bone-renewing and bone-resorbing cells, mostly due to increased osteoblast apoptosis rates (PATSCCHAN et al. 2001). In addition, corticosteroids enhance renal elimination, and reduce intestinal absorption of calcium, resulting in a negative calcium balance. It has been suggested that calcium malabsorption, as well as an increase in urinary calcium excretion, can promote secondary hyperparathyreoidism. It is also noted that glucocorticoid treatment in males antagonizes gonadal function and restrains the osteoanabolic action of sex steroids (REID et al. 1997; PATSCHAN et al. 2001).

1.1.4.2.3. Gastrointestinal disease
Gastrointestinal disease is often unnoticed or simply forgotten as a cause of osteoporosis. Disorders of the gastrointestinal tract have been associated with osteoporosis, most likely as a result of calcium and vitamin D malabsorption. However, gastrointestinal bone diseases have a multifactorial pathogenesis. While genetics play an important role, there are other factors such as malnutrition, systemic inflammation, hypogonadism, corticosteroid therapy in inflammatory bowel disease (IBD), various lifestyle and other factors that may interact and contribute to decreased bone mass.

Postgastrectomy states and small bowel disease have been confirmed to be associated with low BMD in men, whereas large bowel disorders rarely have been related to male osteoporosis (BERNSTEIN a. LESLIEA, 2003; ORWOLL a. KLEIN, 1995).

1.1.4.2.4. Alcohol and tobacco
Alcohol and tobacco use are both independently associated with an increased occurrence of osteoporotic fractures (ANDERSON a. COOPER, 1999). Tobacco-related bone loss is directly connected to smoking duration and quantity. The mechanism may be a combination of decreased body weight, reduced calcium absorption, decreased sex steroid levels, and a direct toxic effect on bone metabolism. Alcohol consumption is inversely connected to bone density in men (ORWOLL a. KLEIN, 1995) and osteoporotic fractures are common in
alcoholic men (KELEPOURIS et al., 1995; PERIS et al., 1995). Alcohol intake has been accompanied with decreased bone formation in humans and experimental animals (KANIS, 1994).

1.1.4.3. Idiopathic Osteoporosis

Some previous reports stated that the age range of male patients with idiopathic osteoporosis varies from 20 to 86 years of age (KELEPOURIS, et al., 1995; FRANCIS et al., 1989; JOHANSSON et al., 1996). However, idiopathic male osteoporosis has been defined as low bone mass occurring in men aged less than 70 years, when all the possible secondary causes of osteoporosis have been excluded (BILEZIKIAN, 1999; VAN POTTELBERGH et al., 2003). Very few studies have concentrated on idiopathic osteoporosis in men, but it is likely that the pathogenesis is multifarious. The involvement of genetic factors to the pathogenesis of idiopathic male osteoporosis remains unknown.

Osteoporosis in these patients commonly manifests as clinical vertebral fractures over a period of 5-10 years, associated with a loss of height, and in severely affected persons unilateral or even bilateral hip fractures (KHOSLA, 1997).

Biomarkers of bone turnover in these cases are usually normal (VAN POTTELBERGH et al., 2003), although bone histomorphometry in these patients indicates decreased bone formation (MARIE, et al., 1991; JOHANSSON et al., 1997). This finding is consistent with previous histomorphometric study in men with senile osteoporosis (CLARKE et al., 1996).

Male idiopathic osteoporosis is in the majority of patients characterized by a low bone turnover state and differs greatly from female postmenopausal osteoporosis, (JOHANSSON et al., 1997; MUNDY et al., 1999,).

The pathogenesis of idiopathic osteoporosis in men at the cellular level is not fully understood. Nevertheless, alterations in estrogen levels and disturbances in the growth hormone (GH) /insulin-like growth factor (IGF) system have been proposed as etiologic factors (PERNOW et al., 2009).
1.2. Bone structure

1.2.1. Anatomy and chemical composition

Bone is a complex, highly organized and specialized connective tissue, that has many functions, including protecting vital organs from trauma, providing mechanical support for soft tissues, providing attachments for muscles that allow them to acts as levers, serving as a calcium storage, and supporting hematopoiesis (WOOLF a. DIXON, 1998; ERBEN, 2005).

Bones can be classified as one of the following types on the basis of external shape: long, short, flat, irregular. The other two bone categories are classified as sesamoid and pneumatic bones (SALOMON et al., 2008).

Long or tubular bones (Ossa longa), such as the humerus, tibia, and femur, have two epiphyses with a midshaft (diaphysis) and a metaphysis (developmental zone) between them (TORTORA, 2008). The epiphysis and the metaphysis are in growing bone separated by the epiphyseal cartilage (the growth plate). The epiphyseal end of the bone is covered with a cartilage layer (JUNQUEIRA a. KELLEY, 1992). Carpal and tarsal bones are classified as short bones (Ossa brevia), because they are almost equal in length and width. Flat bones (Ossa plana), such as the skull bones, scapulas, sternum, ribs, and ilium, provide mechanical protection for the internal organs and sites for muscle attachment. Bones like the vertebral bodies and the calcaneus are defined as irregular bones (Ossa irregularia). Sesamoid bones (Ossa sesamoidea), such as the patella, are occurring in tendons protecting them from excessive wear and tear. Pneumatic bones (Ossa pneumatica) such as the maxilla, sphenoid, ethmoid bone are found only in the skull and contain large air spaces lined with epithelium.

There are two types of bone tissue, cortical and trabecular. The harder external surface, or cortex, is composed of compact bone (cortical bone) and the sponge-like, inner region of bone, that are braced by narrow plates or trabecula, called trabecular (spongy or cancellous) bone (TORTORA, 2008). The external surface of the cortex is covered with periosteum, which has a lining of osteogenic cells. Both compact and cancellous bone tissue consist of bone structural units (BSU), typical structural elements that are created as result of bone remodeling activity (ERBEN, 2005). Cortical bone is composed of cylindrical BSUs - Osteons or Haversian systems, each having a canal in the center (the Haversian canal) that includes blood vessels and nerves. The Haversian canal is surrounded by bone layers surrounding, called lamellae and they contain collagen fibers arranged in thin sheets (JUNQUEIRA a. KELLEY, 1992).
Osteons are connected with the marrow cavity, the periosteum and with each other through transverse or oblique Volkmann's canals. The inside of the cortex is covered with the endosteum. The structural unit in spongy bone is called a trabecular osteon or hemi-osteon, alluding to its shape which can be considered as a half of an osteon (ERBEN, 2005). The trabeculae are consisted of delicate bars and sheets to give the bone maximum strength. The ratio of trabecular to cortical bone differs by skeletal site. Vertebral bodies consist of about 55-75% of cancellous bone, whereas long bones are composed mainly of cortical bone (EASTELL et al., 1990).

The bones are composed of: extracellular matrix, minerals, and several types of bone cells. Under physiological conditions, bone tissue contains approximately 8% water and 92% dry weight (JUNQUEIRA a. KELLEY, 1992; ERBEN, 2005). The organic bone matrix represents about 35% of total dry weight of bone and consists of collagen fibers and non-collagenous proteins. There are several types of collagen in bone, but adult bone is mainly composed of type I collagen which represents more than 90% of the matrix components. This fibrous organic matrix gives bone its resistance to tractional and torsional forces (WOOLF a. DIXON, 1998). The non-collagenous proteins that compose only ten percent of the organic matrix of bone, include proteoglycans, glycoproteins, γ-carboxyglutamic acid (Gla)-containing proteins (osteocalcin), some of the growth factors and plasma proteins.

The inorganic component of the matrix consists mainly of calcium and phosphorus, which comprises about 65% of the dry weight of bone matrix. These minerals form hydroxyapatite crystals (Ca\textsubscript{10} (PO\textsubscript{4})\textsubscript{6}(OH) \textsubscript{2}), that lie closely to the orientation of the collagen fibrils and provide bone its hardness and strength for bone (JUNQUEIRA a. KELLEY, 1992; ERBEN, 2005).

1.2.2. Bone cells
Bone is composed and maintained by three characteristic cell types: osteoblasts, osteoclasts, and osteocytes.

Osteoblasts are cuboidal mononuclear cells, derived from pluripotent bone marrow stromal stem cells. They contain a large amount of rough endoplasmic reticulum and Golgi apparatus
and are characterized as bone-forming cells. Osteoblasts produce type I collagen and form an unmineralized bone matrix called osteoid. These cells have receptors for parathyroid hormone (PTH), vitamin D and estrogens. In addition, osteoblasts produce alkaline phosphatase, an enzyme that plays a key role in the mineralization process (ROBLING et al., 2006). They also synthesize a wide variety of noncollagenous proteins such as osteocalcin, bone sialoprotein, osteopontin, osteonectin, as well as prostaglandin E2 (PGE2) and collagenase (WOOLF a. DIXON, 1998). They are usually positioned at the surface of the existing matrix and deposit fresh layers of matrix, mostly composed of type I collagen. While some osteoblasts stay unbound at the surface, the others slowly become embedded in the unmineralized matrix, as the matrix is rapidly transformed to hard bone matrix by deposition of calcium phosphate crystals (JUNQUEIRA a. KELLEY, 1992). These osteoblasts remain trapped in the lacunae, go through morphological change and develop into osteocytes.

Osteoclasts are large multinucleated lining cells originating from hemopoietic stem cells in the bone marrow. These cells are believed to derive from a stem cell precursor of the monocyte/macrophage lineage (WOOLF a. DIXON, 1998). Osteoclastogenesis begins when a hematopoietic stem cell is stimulated to produce mononuclear cells. Subsequently, these mononuclear cells become committed preosteoclasts they are entering into the blood stream. Some of the circulating preosteoclasts adhere to bone and fuse together into osteoclasts (WOOLF a. DIXON, 1998; ROBLING et al., 2006). Osteoclasts resorb mineralized bone by attaching to the bone surface and secreting hydrogen ions through the ruffled border by a proton pump, that dissolve bone mineral. After that, they release lysosomal enzymes, including metalloproteinases and cysteine proteinases, by exocytosis to degrade organic bone matrix (KANIS, 1994; ERBEN, 2005).

Osteocytes are the small cells within bone matrix that play an important role in bone renewal. These cells are derived from bare inactive osteoblasts, which are incorporated into the matrix and terminally differentiated (ROBLING et al., 2006). Osteocytes are connected with other osteocytes and osteoblast-derived bone-lining cells, which are found on the bone surface (SEEMAN a. DELMAS, 2006). They communicate with one another via gap junctions on filamentous cell projections that pass through a fluid-filled lacuno-canalicular network. These canaliculi are supposed to be accountable for the response of bone to mechanical stimuli (SKERRY et al. 1989). Additionally, it has been speculated that osteocytes and bone-lining cells play an essential role in bone adaptation to mechanical loading, ‘sensing’ physical
strains and initiating an appropriate modeling or remodeling response via the production of a cascade of chemical messengers (ERBEN, 2005; ROBLING et al., 2006). In cortical bone, osteocytes are arranged circumferentially around the concentric bone lamellae, whereas in cancellous bone they lie parallel to the axis of the collagen fibres.

1.2.3. Bone modeling and remodeling
Bone is, contrary to what is commonly believed, a metabolically active tissue. The cellular processes, responsible for the adaptation of the bone structure are modeling (construction) and remodeling (reconstruction). The ability of skeleton to adjust its mass and architecture to its mechanical environment is brought about by continuous bone resorption and bone formation. The purpose of the modeling process is to attain a high peak bone mass and to optimize strength during periods of growth and the purpose of the remodeling process is to maintain bone strength during life (SEEMAN a. DELMAS, 2006). If these continuous mechanisms take place at different locations, the bone morphology is altered. Frost described this as modeling (FROST, 1990).

Bone modeling occurs during the period of growth and it produces a change in the size and shape of bone when new bone is accumulated without previous resorption (SEEMAN, 2008). It refers to the geometric sculpting of bone by the independent actions of osteoclasts and osteoblasts, much like a sculptor might model clay.

In the adult skeleton, the bone is subject to continuous process of bone turnover during life, called bone remodeling. Remodeling refers to the coordinated action of osteoblasts and osteoclasts which take place on the trabecular surfaces, or in cortical bone, in Haversian systems (Figure 1). Old bone is replaced by new, trabecular micro damage is repaired and the strength of skeleton is adapted in proportion to the mechanical stress to which it is exposed (WOOLF a. DIXON, 1998).

During the remodeling process, osteoblasts and osteoclasts act together in teams and establish a temporary anatomic structure, a basic multicellular unit - BMU (ROBLING et al., 2006). Osteoclasts are always trailed by osteoblast teams, and the whole structure moves as a single entity.
Therefore, bone resorption and formation are closely linked to each other in most cases, at least under normal physiological circumstances. Resorption of bone by osteoclasts is estimated to last about three weeks and bone formation by osteoblasts is more protracted than the resorptive and takes about three months (ROBLING et al., 2006).

Figure 1. Bone remodeling cycle (SEEMAN a. DELMAS, 2006).

Bone remodeling is regulated by various systemic and local factors, which involve both the osteoblast and osteoclast cell lineages, exerting their effect on the replication of undifferentiated cells, the recruitment cells and the differentiated function of cells (HILL, 1998). Important systemic regulators include estrogens, androgens, and progesterone, but in large part by two major calcium-regulating hormones, parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D. A third hormone, calcitonin, which can reduce bone resorption, may be significant in skeletal development but seems to play minor role in physiologic calcium regulation in adult humans. It is an effective suppressor of bone resorption and is used clinically in the prevention and treatment of osteoporosis. The local factors are synthesized by bone cells and comprise growth factors and cytokines (RAISZ, 1999).
1.3. Methods for investigating bone density

Fractures in the elderly are often caused by weak and osteoporotic bone, and in order to intervene it is first necessary to assess the fracture risk. Measurement of bone mineral density is of central significance to fracture prevention because different measures of bone density have been shown to be an important determinant of bone strength and a strong predictor of the future risk of fractures in both men and women (DAMILAKIS et al., 2007).

Several non-invasive methods have been developed to measure bone mass. The most common methods used are dual energy X-ray absorptiometry (DXA), quantitative ultrasound (QUS) and quantitative computerized tomography (QCT). Furthermore, peripheral DXA (pDXA), magnetic resonance imaging (MRI), peripheral QCT (pQCT) and micro-computed tomography (µCT) have been used to investigate the bone strength parameters.

1.3.1. DXA

Introduced in the late eighties (CULLUM et al., 1989), dual X-ray absorptiometry (DXA) is currently the most widely used bone densitometry technique (DAMILAKIS et al., 2007). Consequently, the WHO reference standards for osteoporosis and osteopenia are based on bone mineral density measurements made by DXA (KANIS a. GLÜER, 2000). DXA machines permit to measure the amount of mineral in the entire body, femoral neck, and spine fast and reliably. Furthermore, DXA can precisely measure lean body mass and fat body mass. It works in a similar fashion compared with an older technique, dual-photon absorptiometry (DPA), but uses an X-ray source instead of a radioactive isotope. This procedure is superior, because the radiation source does not decay and the energy stays constant over time, resulting in shorter examination time and improved precision and accuracy (KELLY et al., 1988).

DXA uses the differential attenuation of two X-ray beams at different energies to calculate bone mineral content and soft tissue composition in the scanned region.

The first generation of modern DXA scanners used a pencil-beam type of radiation that took 5-10 minutes to scan a patient’s hip or spine. This older pencil beam DXA technology is being replaced by systems using newer fan beam scanners, that provides shorter scan times and improved imagine quality (DAMILAKIS et al., 2007).
The advantages of DXA are the low radiation exposure, good measurement precision and a short scan time. In DXA scans, precision is usually shown as a coefficient of variation (CV). For in vivo scans, a precision in the range of 1 to 2.5% has been reported (CUMMINGS et al., 2002).

One of the main disadvantages of DXA is its inability to distinguish between cortical and trabecular bone (SVENDSEN et al., 1995, LOCHMULLER et al., 2001). In addition, DXA is also affected by bone size because it does not embrace three-dimensional aspects. Therefore, if a large and small bone have the same volumetric BMD (vBMD g/cm3), the larger one will falsely have a greater BMD (CUMMINGS et al., 2002). The important drawback of fan beam DXA is incidence of magnification error when the fan beam does a single sweep across the patient. This decreases the accuracy of fat distribution measurement, while does not affect the measurement of BMD (ABRAHAMSEN et al., 1995).

1.3.2. Peripheral DXA
Peripheral DXA (pDXA) is specially designed for measurement of bone density at peripheral sites, such as the forearm or calcaneus. This process is due to smaller size of the device, portable, cheaper and easier to use than table DXA systems (SIRIS et al., 2001).

A current disadvantage of pDXA is a lack of standardization between different manufacturers and between pDXA and whole body DXA. At present, a T-score below -2.5 is used as a value to determine osteoporosis on all BMD measurements, despite the fact that very few studies have investigated the relationship between pDXA and DXA of total body, hip, and spine. The measurements might also be deceptive, because there are differences in age-related bone loss at various skeletal sites (FAULKNER et al., 1999; GRAMPP et al., 1997).

Moreover, various pDXA models use diverse normative/reference databases, differing in region of interest (ROI) definition, scan acquisition protocols, calibration procedure, and software (GRIGORIAN et al., 2002).

1.3.3. QCT
Quantitative computed tomography (QCT) is a unique method in that it provides for true three-dimensional imaging and reports BMD as true volumetric density measurements
(g/cm³). This quality makes it valuable when examining BMD in growing individuals and adolescents, in whom DXA scanners might miscalculate the true bone density because of growth-related variation in bone size (LANG et al., 1998). It also enables separate measurements of the trabecular and cortical bone compartments, increasing the competence to determine specific effects that certain conditions or pharmaceutical treatments might have on bone structure (ADAMS, 2009). Another advantage of QCT is its precision, especially for assessment of bone mass and structure (BRAILLON, 2002).

The main disadvantages of QCT scanners in comparison to DXA are the higher radiation exposure to patients and relatively high costs. Therefore, they are not widely used in clinical practice. An additional drawback of QCT is that the WHO has not identified thresholds for identifying osteoporosis using QCT measurements as it has for DXA measures. This technique is currently mainly used as a research device (ADAMS, 2009).

1.3.4. pQCT
Peripheral QCT is employed to determine bone mineral density at peripheral sites of the body, such as the radius, phalanges and tibia. Peripheral QCT of the forearm at the distal radius have been demonstrated to predict hip fractures, but not vertebral fractures, in postmenopausal women (ADAMS, 2009).

The benefits of using the peripheral QCT comprise that the method is relatively inexpensive, easier to use, portable and the radiation dose to which patients are exposed is insignificant (ADAMS, 2009).

1.3.5. MRI
Magnetic resonance imaging (MRI) has been recently introduced and appears to be a method for investigating trabecular bone micro-architecture and the structural components of bone (KRUG et al., 2008). High-resolution MRI can examine the cancellous bone network in both two and three dimensions. This enables to analyze the parameters such as the bone volume/total volume ratio, trabecular thickness, trabecular number and trabecular separation of the bone that is being imaged, which can be used to assess for osteoporosis (DAMILAKIS et al., 2007).
This technique also allows depicting the trabecular bone structure and biomechanical strength of bone specimens both in vitro (LINK et al., 1998) and in vivo (MAJUMDAR et al., 1999).

Although the both methods, MRI and QCT can assess trabecular bone micro-architecture, MRI has the advantage of avoiding ionizing radiation and allows for multi-planar image acquisition (KRUG et al., 2008). The major drawbacks of MRI include the high cost and the time required to perform the test.

1.3.6. QUS

Quantitative ultrasound (QUS) is a promising technique and has recently been widely applied for the characterization of bone tissue. The QUS method has been shown to be a useful tool for the assessment of bone mineral status and fracture risk.

This technique employs sound waves rather than radiation to analyze bone density and bone strength properties (CUMMINGS et al., 2002). QUS is based on differential reflections and attenuation of ultrasound beams as they traverse bones. This device can measure the speed at which the ultrasound beam passes through bone. This is called Speed of Sound (SOS) expressed in m/s. The other parameter which is assessed is broadband ultrasound attenuation (BUA) expressed in dB/MHz. Measurement of BUA operates by transmitting a broadband ultrasound wave through the bone and measuring the decrease of intensity at different frequencies. SOS is affected by bone density and elasticity, whereas BUA is related to bone structural parameters and also to bone density.

It has been showed that calcaneal QUS examination foretells the risk of hip fracture and non-spine fractures in older men (BAUER et al., 2007), and osteoporotic fractures, particularly hip fractures, in women (GUESSOUS et al., 2008; HUOPIO et al. 2004). The ultrasound assessments of the calcaneus appear to be highly reliable and precise to measure longitudinal changes in BMD over time, even in the elderly (ZOCHLING et al., 2004). Nevertheless, DXA still remains the standard procedure for evaluating the fracture risk because it has been verified in several populations and has been well established in both clinical and research settings, with reported normative data that guarantees the precision and reliability of the results (BAUER et al. 2007). Therefore, a better standardization of instruments is required if QUS is to be used for the diagnosis of osteoporosis (ZOCHLING et al., 2004).
Quantitative ultrasound (QUS) equipment has many advantages because it is inexpensive, does not cause ionizing radiation exposure, and is portable. A disadvantage is that it does not discriminate between trabecular and cortical bone (SCHOENAU et al. 2004; VAN RIJN et al., 2003).

1.3.7. μCT
Micro-computed tomography (μCT) is an emerging technique to image and quantify bone in three dimensions currently under development for bone measurements. It is a non-destructive, fast and precise procedure that can accurately visualize and quantify cancellous bone microstructure. However, μCT based measurement of bone mineral density has not been thoroughly investigated. Particularly, the effects of varying imaging parameters, such as tube voltage (kVp), current (μA), integration time (ms), object to X-ray source distance (mm), projection number, detector array size and imaging media (surrounding the specimen), have to be evaluated (NAZARIAN et al., 2008).
1.4. Physical activity, obesity, diet restriction and BMD

1.4.1. Exercise and bone

Physical activity is considered as one of the significant regulators of bone mineral density. Furthermore, to comprehend the mechanism underlying the effect of physical activity on bone, one must look into the theories on impact of mechanical loading on bone.

According to the many studies using animal models, primarily rats and birds, it is well recognized that the important stimulus for osteogenesis is bone deformation or strain, which is applied to bone by muscle contractions or dynamic ground reaction forces (RUBIN a. LANYON, 1984; TURNER, 1991; ROBLING et al., 2002).

Strain ($\Delta l/l$) is characterized as the degree of deformation ($\Delta l$) in relation to the original dimensions ($l$) and is in general expressed in microstrains. One microstrain is equivalent to a deformation of 0.0001%. The most effective load to increase BMD is usually produced by activities with high strain amplitude, a high strain rate, and where the strain is applied at various angles. These activities are referred to as osteogenic activities (OA). The duration of the activity appears to be of less significance, and a sufficient amount of rest periods between stimuli will increase the osteogenic response, seemingly due to a reduced stimulus accommodation (RUBIN a. LANYON, 1985; LANYON et al., 1986; LANYON, 1992; ROSS et al., 1993; RAAB-CULLEN et al., 1994).

When external forces exert their effect on the skeleton, they induce bone strain. Subsequently, strain produces a hydrostatic pressure change inside the canalicular network, connecting the osteocytes, generating shear stress on the bone cells (TURNER a. ROBLING, 2003). Osteocytes and osteoblasts are very sensitive to shear stress and react by initiating a cascade of reactions inside the cell. Concisely, these reactions involve increase of intracellular calcium, paracrine/autocrine secretion, expression of growth factors and ultimately bone matrix production (TURNER a. ROBLING, 2003; TURNER a. ROBLING, 2005). The mechanism by which cells convert mechanical signals into electrical and/or biochemical responses is called mechanotransduction.

The mechanism behind mechanotransduction is still not fully understood but is supposed to comprise four phases (TURNER a. PAVALKO, 1998):
1) Mechanocoupling: transduction of external force applied to the bone into a local mechanical signal that is detected by a sensor cell.

2) Biochemical coupling: transduction of a local mechanical signal into a biochemical signal that leads to changes in gene expression and/or protein activation.

3) Cell-to-cell communication: transmission of a signal generated in the sensor cell to the effector cell.

4) The effector cell response: either production or removal of bone tissue to cause appropriate architectural changes.

According to Wolff’s law, a theory developed in the 19th century, the changes in bone function are followed by changes in internal architecture and external conformation (WOLFF, 1892).

In 1987 Harold Frost proposed the more modern Mechanostat theory, based on the same principals, defining a model by which bone adapts to external loading or disuse under influence of hormonal and biochemical substances (FROST, 1987; TURNER, 1991; ROSS et al., 1993; FROST, 1999).

This theory suggests that there are two thresholds values for strain below or above which bone adaptation will be turned on. The lower range is termed the minimum effective strain for remodeling (MESr) and below this, there is an inadequate stimulus and resorption will prevail, resulting in loss of bone. The upper range is named the minimum effective strain for modeling (MESm), and above this threshold, the modeling is provoked to add more bone.

The strain values between these two thresholds defined area is called the physiological loading zone, and in this zone remodeling is in a kind of balanced state, attuned by sufficient level of strain stimuli where there is no change in bone mass.
The skeleton’s sensitivity to mechanical loading may vary during different periods in life. Although the mechanism involved remains unknown, it may be connected with the fact that during growth, the bone surfaces are covered with greater number of active osteoblast than after puberty (TURNER a. ROBLING, 2005).

1.4.2. Types and modalities of exercises
Physical exercise programs have been widely used as part of osteoporosis prevention and treatment (PAJAMÄKI et al., 2003). The most appropriate exercise modality and intensity, capable of eliciting the most efficient osteogenic response still remains unclear. Many authors have investigated the effect of exercise and mechanical load on skeleton (PENG et al., 1994; BARENGOLTS et al., 1994; HONDA et al., 2003). Various types of exercise programs have been suggested, including running, walking and swimming (PENG et al., 1994; BARENGOLTS et al., 1994; HART et al., 2001; HONDA et al., 2003). A lot of studies have implied that dynamic high-impact exercises and resistance training are more beneficial to bone tissue, generating greater strain than walking (HONDA et al., 2003; NOTOMI et al., 2000).

The influence of physical activity on bone strength depends on age and to some extent, on gender. Many reports regarding the relation between exercise and BMD in puberty and middle-aged period, have suggested that moderate exercise can increase BMD, whereas prolonged, excessive exercise as in marathon runners, negatively affects BMD (MYBURGH et al., 1993; WELTEN et al., 1994, GOTO et al., 1995; FRIEDLANDER et al., 1995).

Even though many authors suggest that exercise is effective in preventing osteoporosis in humans, a definite conclusion has not yet been reached, because different types, periods, frequencies, amounts and intensities of exercises have been proposed. Running exercise is recommended as one of the best methods to avert osteoporosis because of its effectiveness in maintaining and increasing bone mineral density (BMD).

Moreover, high-impact activities, such as running, are reported to be more beneficial in enhancing or maintaining bone mass than non-weight-bearing exercise, such as road cycling, in men aged 20–59 years (RECTOR et al., 2008). Running activity is one of the preventive methods that are available to all; nevertheless, it is difficult to establish what exactly moderate
conditions are. Furthermore it remains problematic that, while running exercise is locally effective in bones of loaded sites, it has shown less, if any, efficacy on bones of the whole skeleton.

1.4.3. Bone and obesity
While obesity is accompanied with increased risk of many chronic diseases including cardiovascular disease, diabetes, hypertension, and cancer, there is evidence suggesting that body weight positively correlates with bone mass. According to various studies, obesity may protect against osteoporosis. In fact, higher body weight and body fat have been shown to increase bone mass, bone mineral density and content (DALEN et al., 1975; ALBALA et al., 1996; COMPSTON et al., 1992; RICO et al., 1991; MAZESS et al., 1990). However, it still remains unknown whether it is fat mass (RAVN et al., 1999; RIBOT et al., 1994; PLUIJM et al., 2001), lean tissue mass (RAVN et al., 1999; KHOSLA et al., 1996; BAKKER et al., 2003), or total weight (RAVN et al., 1999; EDELSTEIN a. BARRETT-CONNOR, 1993; GOSSAIN et al., 1999) that is associated with increased bone mass.

There are two methodological grounds that can explain the difficulty in clearly defining the relations of fat mass, lean tissue mass and weight with bone mass. Firstly, fat mass and lean body mass are highly connected, and small samples that do not present broad variance in adiposity make it hard to differentiate the effects of fat mass from those of lean tissue mass. Secondly, variations in the particular bone mass parameters that are employed, such as bone mineral content (BMC), areal bone mineral density (BMD), or assessed volumetric bone mineral density (bone mineral apparent density, BMAD), and in the population studied (estrogen-replete or estrogen-deficient women; males or females, etc.) make it difficult to collate results across studies (KHOSLA et al., 1996; REID, 2002).

1.4.4. Bone, exercise and diet restriction
Most of the studies researching the influence of either physical activity or food restriction on the rat skeleton have been performed with female rats. Nonetheless, as the sex hormones have a considerable impact on the growth pattern of rat bone (KIM et al., 2003), the effects of physical exercise and dietary restriction on the animal skeleton may, therefore, be gender specific. Furthermore, most researchers commenced their experiments (exercise or food restriction) when the animals were very young and rapidly growing (PAJAMÄKI et al., 2003;
NOTOMI et al., 2000). However, an early study in male adult rats has found negative effect of dietary energy restriction on bone mineral content (LEE et al., 1986). This is in accordance with a report, showing that dietary-induced reduction of body mass in women was accompanied by significant decrease in total body BMD (COMPSTON et al., 1992).

1.4.5. Physical activity and BMD in men
Several cross-sectional studies have shown that male athletes engaged in weight-bearing activities have higher BMDs than sedentary controls (WITTICH et al., 1998, FREDERICSON et al., 2007, CALBET et al., 1998; CALBET et al., 1999; DUCHER et al., 2006) or athletes engaged in non-weight-bearing sports, such as swimming (MAGKOS et al., 2007; MOREL et al., 2001) or cycling (NICHOLS et al., 2003; RECTOR et al., 2008).

However, one randomized controlled study in men, using weight lifting as the intervention (FUJIMURA et al., 1997) failed to demonstrate any significant differences in BMD between weight lifters and controls, after a period of four months. The possible explanation for such an outcome might be found in a non-dynamic type of the activity, or duration, as it may be that the intervention time is too short period to detect any changes in BMD by DXA. In addition, the results from two longitudinal observational studies (DALY a. BASS, 2006; DELVAUX et al., 2006) as well as retrospective studies with wide age span indicate that weight-bearing physical exercise and an active lifestyle seem to be connected with higher BMD and reduced bone loss at weight-bearing sites in men (NGUYEN et al., 2000; LYNCH et al., 2007).

1.4.6. Rodents as a model for human osteoporosis
The most frequently employed animal model for osteoporosis studies is the rodent (BARLET et al., 1994). For instance, the ovariectomized (OVX) rat shows most of the characteristics of human postmenopausal osteoporosis (TURNER, 2001). Rodents are indispensable for preliminary screenings, assessment of efficacy and toxicity of a new pharmacological agent or therapeutic modality, followed by confirmation in other species, before commencing clinical trials in human patients (AERSSENS et al., 1998). Rodents provide many benefits in biomedical research; they are inexpensive, easy to house, and modern society is accustomed to the role of rodents for use in scientific investigations. Since the rodent has been used so widely in all types of research, much is known about bone turnover and the effect of diet on
this process. Cortical bone loss and decreased bone mechanical properties are well documented in aging rat and mouse bone.

Because older animals more accurately mirror the target population for proposed osteoporosis therapy, the very aged rat model (30-month old) may be an even better choice as a cost effective animal model (GAUMET et al., 1996).

Furthermore, rats demonstrate significant increase of cortical porosity in response to immobilization (SIETSMA, 1995). Some authors (PENG et al., 1994) have suggested that the mechanical strength of the rat femoral neck was a sensitive indicator of bone loss induced by ovariectomy, orchidectomy or immobilization.

The majority of the in vivo studies investigating the effects of exercise on the rodent skeleton used the flatbed treadmill, which is capable of producing high bone strains of normal coordinated activity (BARENGOLTS et al., 1994; HAGIHARA et al., 2005; MOSEKILDE et al., 1994). Thus, to use exercise therapy efficiently will require a better understanding of the influence of loading and exercise on bone modeling and remodeling, and the development of appropriate exercise regimens for various bones.

In this study, was employed voluntary wheel running exercise, which is likely to inflict a more unusual pattern of strain on the rat skeleton than the flatbed treadmill.

As it is increasingly evident that osteoporosis is common in men with advancing age, there was an uncertainty about the most acceptable, convenient animal model for studying male osteoporosis. According to several reports (ERBEN et al., 2000; BANU et al., 2002; WANG et al. 2001) male Sprague-Dawley rats appear to be better suited for studying the age-related bone loss than Fischer 344 rats.
1.5. Aims of the study

Intervention studies examining the modulating effect of exercise on age-related bone loss in skeletally mature male rats are scarce. Therefore, it was the aim of the present study to investigate the interaction between two running exercise modalities (voluntary running wheel and forced treadmill exercise) and dietary restriction with age-related bone loss in the appendicular skeleton of male Sprague-Dawley rats.

We hypothesized that physical exercise would at least partially prevent the age-related bone loss observed in sedentary control rats.
2. MANUSCRIPT

TITLE PAGE

2.1. Interaction between exercise, dietary restriction, and age-related bone loss in a rodent model of male senile osteoporosis
Interaction between exercise, dietary restriction, and age-related bone loss in a rodent model of male senile osteoporosis

Marko Bodnar¹, Monika Skalicky¹, Andrus Viidik², and Reinhold G. Erben¹

¹ Institute of Physiology, Pathophysiology and Biophysics, Dept. of Biomedical Sciences, University of Veterinary Medicine, Vienna 1210, Austria
² Institute of Anatomy, University of Aarhus, Aarhus, Denmark

Running title: Exercise and dietary restriction as modulators of senile osteoporosis

Abstract
Background: The pathophysiology of age-related bone loss, and whether age-related bone loss can be prevented by exercise are still a matter of debate. Objective: It was the aim of this study to investigate the long-term effects of exercise and mild food restriction on bone mineral density (BMD) and on bone geometry in the appendicular skeleton of aging male rats. Methods: Male Sprague-Dawley rats were studied from 5 to 23 months of age. The rats were divided into 4 groups: Baseline (BL), free access to food and running wheels (RW), fed to pair weight with the RW group (PW), and sedentary control animals with free access to food (SED). All rats were housed individually. Volumetric BMD and geometry of femurs and tibiae were assessed by peripheral quantitative computed tomography (pQCT). In addition, the tibial shafts were analyzed by cortical bone histomorphometry. Results: At the end of the experiment RW and PW had similar body weight. The body weight of SED rats was 31% higher compared with RW rats. pQCT analysis of femurs and tibiae as well as histomorphometric analysis of the tibial shaft showed that dietary restriction resulted in an enlargement of the marrow cavity and cortical thinning at the femoral and tibial shaft relative to the RW and SED groups. Voluntary running exercise provided no additional protection against age-related bone loss when compared with the 31% heavier SED control rats. Neither exercise nor increased body weight in SED animals could completely prevent age-related bone loss between 19 and 23 months of age. Conclusion: We conclude that dietary restriction had clear untoward effects on BMD and bone geometry, and that running wheel exercise provided partial protection but could not prevent age-related bone loss.

Key words: Age-related bone loss – Exercise – Osteoporosis – Dietary restriction – Cortical bone

Corresponding author:
Reinhold G. Erben, M.D., D.V.M.
Institute of Physiology, Pathophysiology and Biophysics
Dept. of Biomedical Sciences
University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
Phone +43-1-250 77 4550, Fax +43-1-250 77 4599, E-mail Reinhold.Erben@vetmeduni.ac.at
INTRODUCTION

With the rapid increase of the elderly population in most Western and high developed societies, some age-related diseases have received much attention, and have become a major public health problem. Osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a subsequent increase in susceptibility to fracture [1]. This disease is attributed to age, sex steroid deficiency and certain medical conditions, and has immense socio-economic significance. Although postmenopausal osteoporosis has been the main focus of osteoporosis research, it is increasingly evident that osteoporosis in elderly men is a frequent and severe condition [2-5].

Age-related bone loss is a primary factor in osteoporosis and is thought to be the result of an imbalance in the volumes of bone resorbed and formed in each focal basic multicellular bone remodeling unit. The exact cause of this imbalance is unknown. Endocrine alterations such as sex hormone deficiency or secondary hyperparathyroidism may play an important pathophysiological role in age-related bone loss [4]. However, because physical activity declines with increasing age, age-related bone loss may simply reflect, at least in part, a normal adaptation of bone to decreased biomechanical stimuli.

Age-related bone loss is a very well documented phenomenon in aging male rats. For example, male Sprague-Dawley rats show a continuing decline in lumbar vertebral and proximal tibial trabecular volumetric bone mineral density (BMD) between 9 and 27 months of age, despite unchanged levels of circulating testosterone [6]. Growth of the appendicular skeleton slows down markedly at around 5 - 7 months of age in male rats [7], and male rats reach their peak bone mass at about 12 months of age [8]. In one of our earlier experiments, male Sprague-Dawley rats lost about 50% of trabecular BMD, and about 70% of cancellous bone volume in the proximal tibial metaphysis between 5 and 23 months of age [9]. Although we found an approximately 50% decline in tibial cancellous bone volume between the ages of 13 and 22 months in male Fischer 344 rats [10], age-related bone loss appears to be generally more pronounced in male Sprague-Dawley compared with Fischer 344 rats [6;11]. Therefore, aging male Sprague-Dawley rats appear to be an appropriate model to study age-related bone loss.

Intervention studies examining the modulating effect of exercise on age-related bone loss in skeletally mature male rats are scarce. Therefore, it was the purpose of the current study to investigate the interaction between voluntary running wheel exercise and dietary restriction with age-related bone loss in male Sprague-Dawley rats. We started the dietary and exercise interventions at the age of 5 months, when nose-rump length has reached its maximum level, and when bone elongation in the appendicular skeleton has reached very low levels in male Sprague-Dawley rats [12].

MATERIAL AND METHODS

Animal procedures
All animal procedures were approved by the Ethical Committee of the University of Veterinary Medicine Vienna, and the government authorities. One-month-old male Sprague-Dawley rats were obtained from the Research Institute of Laboratory Animal Breeding, Himberg, Austria. The rats were kept in groups of 5 animals per cage with free access to a standard rat chow at 21-23°C, and a 12h:12h light-dark cycle until the age of 5 months, when they were divided into 4 groups (n = 20 for baseline, other groups n = 64 each): (1) BL, baseline animals sacrificed at
the age of 5 months, (2) RW, voluntary exercise in running wheels, (3) PW, sedentary animals fed to pair weight with the RW group, (4) SED, sedentary animals with free access to food. After start of the experiment at 5 months of age, all animals were housed individually in cages. Fifteen animals in each group were killed at 15 and 19 months, the remaining animals (n = 18 – 28 each) at 23 months of age. The animals in the RW group had voluntary exercise in a running wheel attached to the cage. The running distance as well as the running profile (speed, activity time etc.) of the individual animals was recorded throughout the study. The PW animals fed to pair weight with the RW animals received on average of 16 g food per day. This group was designed for the comparison between the effects of achieving the same body weight by voluntary running or by mild food restriction. All animals had ad libitum access to water and food except the PW group. The weight of the animals was recorded weekly in groups 2 and 3, and monthly in group 4. The animals in groups 2-4 were sacrificed by heart puncture under halothane anesthesia at the age of 15, 19 or 23 months. The left hind limbs were removed and stored frozen (at -20ºC) until subsequent analysis.

Peripheral quantitative computed tomography (pQCT)
The frozen hind limbs were thawed. Subsequently, femora and tibiae were carefully stripped of soft tissue, and fixed in 70% ethanol. Volumetric bone mineral density (BMD; mg/cm³), bone mineral content (BMC; mg/mm), and bone geometry of the left femur and tibia were measured by pQCT using an XCT Research M+ pQCT machine (Stratec Medizintechnik, Pforzheim, Germany). Slice thickness was 200 µm. The femur was examined at the midshaft (one slice) and at the distal metaphysis (three slices). BMD values of the distal femoral metaphysis were calculated as the mean over 3 slices. The tibia was examined at the proximal (one slice located 4.5 mm distal from the articular surface) and distal metaphysis (one slice located 2 mm proximal from the articular surface) as well as at the mid-diaphysis located 2 mm proximal to the tiobiofibular junction (one slice). The voxel size was 100 µm, and a threshold of 710 mg/cm³ were used for calculation of cortical BMD.

Cortical bone histology and histomorphometry
After pQCT measurements, the tibial shafts from the 23-month-old rats were embedded in methylmethacrylate, and 200-µm-thick cross-sections were taken 2 mm proximal to the tiobiofibular junction with a precision band saw (Exakt, Norderstedt, Germany). The sections were subsequently ground to a final thickness of 20 µm with the help of the microgrinding system (Exakt) as described [13]. Quantitative bone histomorphometry was performed with an automatic image analysis system (AxioVision, Carl Zeiss) on sections stained with toluidine blue. Total cross-sectional area (CSA), cortical bone area, marrow area, endocortical and periosteal perimeter as well as the number, area, and perimeter of cortical pores were determined. Cortical thickness was measured on 90 radii originating from the center of gravity of the bone cross-section, and was expressed as the mean value of these measurements.

Statistical analysis
Statistics were computed using SPSS for Windows 17 (SPSS, Chicago, IL, USA). The data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan’s multiple comparison test as post hoc test. A p value <0.05 was considered significant for all statistical analyses. The data are presented as the mean ± SEM.
RESULTS

Body Weight
As shown in Fig. 1 the initial body weight of all groups at baseline was comparable.

The nonsignificant trend for lower body weight in the RW group at baseline was caused by adaptation of the rats to running in wheels, which was started shortly before beginning of the study. The RW and PW groups had similar body weight throughout the experiment. The body weight of SED rats continued to increase until 19 months of age. All groups of animals tended to lose body weight between 19 and 23 months of age. However, this effect was statistically significant only in SED rats. At the end of the study, the body weight of SED rats was 31% higher compared with RW rats.

Age-related bone changes over all groups
At the proximal tibial metaphysis and the distal femoral metaphysis, all groups of animals lost trabecular BMD between 5 and 23 months of age (Figs. 2C and 3C). Proximal tibial metaphyseal loss of trabecular BMD was most pronounced in SED and PW rats, but was also present in the RW group. Probably as a compensatory mechanism, cortical-subcortical BMD showed a pronounced increase in tibial and femoral metaphyseal bone in all groups, especially between 5 and 15 months of age (Figs. 2B and 3B). At the proximal tibial metaphysis, total BMD did not differ between 5 and 23 months of age, whereas all groups lost total BMD at the distal femoral metaphysis between the 5- and 23-month time points (Figs. 2A and 3A). At the distal tibial metaphysis, consistent age-related changes over all groups were absent (Fig. 4). Rather, the observed changes in BMD were dependent on body weight and exercise.

At the tibial and femoral diaphysis, all groups of animals showed an age-related increase in cross-sectional area, and a concomitant increase in marrow area in pQCT measurements (Figs. 5D and F and 6D and F). This was also clearly evident from microground cross-sections (Fig. 7A and B). Similar to the metaphyseal region, cortical BMD of the tibial and femoral shaft increased distinctly between 5 and 15 months of age in

Figure 1. Changes in body weight during the experiment. Data are means ± SEM of 15 – 28 animals each. Significant differences (P<0.05) across groups at a given time point are indicated by different letters. Groups marked with the same letter are not significantly different from each other. For example, a group marked with “a” is significantly different from a group marked with “b” or “c”, but not from a group marked with “ab”. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline. SED, sedentary; PW, paired weight; RW, running wheel.
all groups (Figs. 5B and 6B). Cortical BMD declined between 19 and 23 months of age at the femoral shaft in all groups, but not at the tibial shaft (Figs. 5B and 6B).

**Running exercise has beneficial, but dietary restriction detrimental effects on bone**

Despite comparable body weight, RW and PW rats showed distinct differences in BMD and bone geometry. At the end of the study, RW rats had higher trabecular but lower cortical-subcortical BMD at the proximal tibial metaphysis than PW rats (Figs. 2B and C). The most pronounced differences between RW and PW groups were present at the distal tibia and at the tibial and femoral shaft. At the distal tibia, RW rats had higher total, trabecular, and cortical-subcortical BMD than PW rats throughout the study (Fig. 4).

**Figure 2.** The effects of exercise and diet restriction on total BMD (A), cortical-subcortical BMD (B) and trabecular BMD (C) of the proximal tibial metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
At the tibial and femoral shaft, RW rats were characterized by higher total BMD and cortical thickness as well as reduced marrow area relative to PW rats (Figs. 5 and 6). Histomorphometric analysis of the tibial shaft revealed similar cross-sectional area, but higher absolute and relative cortical area and cortical thickness, and lower absolute and relative marrow area in RW compared with PW rats (Fig. 7). It is evident from Fig. 7A that dietary restriction resulted in a pronounced expansion of the marrow cavity in PW rats. Taken together, running wheel exercise had a beneficial skeletal effect especially at cortical bone sites, relative to food-restricted sedentary PW rats with similar body weight.

The comparison between sedentary rats with free access to food (SED group) and diet-restricted sedentary rats fed to pair weight with the RW group (PW group) was generally similar to the comparison between RW rats and PW rats. Relative to SED rats, PW rats had lower total, trabecular, and cortical-subcortical BMD at the distal tibia (Fig. 4), and lower total BMD, cortical thickness, and

**Figure 3.** The effects of exercise and diet restriction on total BMD (A), cortical-subcortical BMD (B) and trabecular BMD (C) of the distal femoral metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
cortical area at the tibial and femoral shaft (Figs. 5 – 7). These results indicate that dietary restriction had negative skeletal effects in this experiment, and that body weight is a modulator of age-related bone loss in male rats, independent of exercise.

The positive skeletal effects of exercise are not superior to those of increased body weight in sedentary controls.

Total BMD of the proximal tibial metaphysis, distal femoral metaphysis, as well as the tibial and femoral diaphysis was not different between SED and RW rats at the end of the study, showing that running wheel exercise did generally not result in more bone compared with SED controls characterized by a 31% higher body weight than RW rats (Figs. 2 - 7).

In fact, increased body weight in SED rats completely prevented the age-related loss of total BMD at the distal tibial metaphysis (Fig. 4). The only site where RW rats showed higher BMD than SED rats was the proximal tibial metaphysis, where trabecular BMD was

Figure 4. The effects of exercise and diet restriction on total BMD (A), cortical-subcortical BMD (B) and trabecular BMD (C) of the distal tibial metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
Figure 5. The effects of exercise and diet restriction on total BMD (A), cortical BMD (B), cortical thickness (C), total cross-sectional area (CSA, D), cortical area (E), and marrow area (F) of the tibial shaft assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
higher in RW compared with SED rats at the age of 23 months (Fig. 2C). It is also interesting to note in this context that neither exercise in RW rats nor increased body weight in SED rats were able to prevent the age-related decline in BMD at the proximal tibial metaphysis and the tibial and femoral shaft, as well as the expansion of the marrow cavity at the tibial and femoral shaft between 19 and 23 months of age (Figs. 2, 5, and 7).

DISCUSSION
The pathophysiology of age-related bone loss, and whether age-related bone loss can be prevented by exercise are still a matter of debate. In this time course experiment, we subjected 5-month-old male Sprague-Dawley rats to 18 months of voluntary running wheel exercise as well as to mild food restriction by feeding to pair weight with the RW group from 5 to 23 months of age. Sedentary animals fed ad libitum were used as controls. We found that dietary restriction clearly had untoward effects on total volumetric BMD and bone geometry at the distal tibial metaphysis and at the tibial and femoral shaft, relative to RW and SED rats. Similar to our data, dietary restriction in aged female rats resulted in an enlargement of the marrow cavity [14]. However, other studies found no negative effect of dietary restriction on bone mass and biochemical markers of bone metabolism in female rhesus monkeys [15], or bone geometry and biomechanical properties in young and senescent male rats [16]. Compared with food-restricted sedentary rats (PW group) with comparable body weight, voluntary running exercise could partially prevent the age-related bone loss especially at the tibial and femoral mid-diaphysis. However, voluntary running did not provide an additional benefit in terms of protection against age-related bone loss relative to ad libitum-fed SED animals characterized by 31% higher body weight. With the exception of the distal tibial metaphysis, neither exercise nor increased body weight in SED animals could completely prevent age-related bone loss between 19 and 23 months of age.

Therefore, our study suggests that disuse per se is not the central mechanism leading to age-related loss of bone mass or bone structural integrity. Rather, other factors must be responsible for the decline in BMD seen in all groups in this study between 19 and 23 months of age. It is still controversial whether the responsiveness of the aged skeleton to mechanical loading is altered. It has been reported that the responsiveness of the aged skeleton is increased [17,18], reduced [19,20], or unaffected [21,22] compared with the younger skeleton. Therefore, it is unclear whether reduced mechanoresponsiveness is involved in age-related bone loss.

To examine the effects of exercise on bone mass or strength, most previous studies have used treadmill exercise on a non-voluntary basis. The results from these studies have been conflicting: Some have shown no change or even decreases in mass or strength [23,24], while others found increased mass and strength [22,25,26]. These conflicting results could be due to the age of the rats, the duration and type of exercise regimes, or the skeletal sites tested. Rat treadmill experiments in which higher speed and longer time intervals were applied showed an improved response in terms of cancellous bone formation and bone mass [27-29].

Despite the marked differences in BMD and bone geometry at the tibial and femoral shaft observed in our study, 3-point bending tests of femurs from the same experiment revealed no difference between RW and PW rats [12]. However, in accordance with our finding that trabecular BMD at the distal femoral and proximal tibial metaphyses declined
Figure 6. The effects of exercise and diet restriction on total BMD (A), cortical BMD (B), cortical thickness (C), total cross-sectional area (CSA, D), cortical area (E), and marrow area (F) of the femoral mid-diaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
precipitously between baseline and the end of the trial, axial compression tests of the distal femoral metaphysis showed a pronounced reduction in bone strength between 5 and 23 months of age in all groups [12].

A comparison of the values for area measurements at the tibial shaft obtained by pQCT (Fig. 5) with histomorphometric measurements at the same site (Fig. 7) shows that the values measured by pQCT were about 10% higher than those measured by histomorphometry. As reported previously [13], the inbuilt software of the pQCT
machine used overestimates area measurements by about 10% which may be due to projection errors. However, this systematic error does not interfere with the comparison between different groups. Overall, the differences between the groups were in very good agreement between pQCT and histomorphometric measurements.

An interesting and unexpected observation in this study was that running wheel exercise did not provide additional protection against age-related bone loss when compared with SED controls rats. Although running wheel exercise reduced the expansion of the marrow cavity at the tibial and femoral shaft seen in all group of rats between baseline and 23 months of age (Fig. 5F and 6F), total BMD and cortical thickness at the tibial and femoral shaft were similar between RW and SED rats at the end of the trial. There are several possible explanations for this finding. First, the higher body weight in SED relative to RW rats leads to increased mechanical loading of the appendicular skeleton, which may in turn protect bone. Second, the increased adipose tissue in SED vs. RW rats may additionally influence bone metabolism by other factors than just increased mechanical loading. Fat tissue may exert beneficial effects on bone due to increased aromatization of androgen to estrogen [30], or high circulating levels of insulin [31], insulin-like growth factor 1 [32], and leptin [33]. Several epidemiological and twin studies have reported fat mass to be an independent predictor of BMD [34-36]. A recent study in young male mice also reported that diet-induced obesity has positive effects on bone properties, and interacts with physical exercise [37]. Third, the sympathetic tone may have been different in RW compared with SED rats. We did not measure circulating glucocorticoid hormones in this study. Therefore, we do not know whether stress levels were different between different groups of rats in our experiment. Nevertheless, it has been shown that the sympathetic tone has a profound influence on bone metabolism, and interacts with leptin in the regulation of bone formation and bone resorption [38;39].

In conclusion, this study has shown that voluntary running wheel exercise had no additional protective effects against age-related bone loss when compared with about 30% heavier SED rats. In contrast, mild food restriction in sedentary animals to reach paired weight with voluntarily exercising rats resulted in a clear detrimental effect on bone mass and geometry at the distal tibia and at the tibial and femoral diaphysis. Although extrapolations from rat experiments to humans always need to be treated with caution, the latter finding may also be of relevance for clinical medicine.

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References


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Dear Prof. Erben,

Thank you for submitting a once-more revised version of your manuscript to "Gerontology". We are pleased to inform you that it has been accepted for publication and passed on to the publishers from whom you will hear shortly.

We hope you will continue to submit work from your group to "Gerontology" in the future!

With kind regards,
Christine Suess
Laboratory of Autoimmunity

Innsbruck Medical University
Peter-Mayr-Straße 4a
A-6020 Innsbruck

Austria

Tel. +43/512/9003/70960
Fax. +43/512/9003/73271
e-mail:gerontology@i-med.ac.at
3. DISCUSSION

There is still no firm evidence that exercise in adult humans increases cortical bone strength by increasing periosteal apposition, reducing endocortical resorption, or increasing endocortical bone formation (SEEMAN, 2002). Therefore, animal experiments are needed to examine the interplay between the skeletal effects of exercise and aging. It was the aim of the present study to examine the interaction between two running exercise modalities (voluntary running wheel and forced treadmill exercise) and dietary restriction with age-related bone loss in the appendicular skeleton of male Sprague-Dawley rats. We subjected male Sprague-Dawley rats to 18 months of voluntary (RW group) and forced (treadmill, TM group) physical exercise as well as to mild food restriction by feeding to pair weight (PW group) with the RW group from 5 to 23 months of age.

One of the reviewers of the manuscript asked us to remove the TM group from the manuscript because the body weight of the TM rats was higher than that of the RW and PW groups, so that a clear distinction between the effects of body weight and treadmill exercise could not be made. Therefore, the TM group is not part of the manuscript any more. The complete results, including the data of the TM group are shown in the Appendix.

The results of the current experiment have shown that voluntary running exercise can partially prevent age-related bone loss, especially at the tibial and femoral mid-diaphysis. However, exercise did not provide additional protection against age-related bone loss compared with higher body weight in sedentary animals. Considering the interaction between physical activity and aging, our study has shown that both running wheel and treadmill exercise did not completely prevent age-related bone loss between 19 and 23 months of age. In addition, we found that effects of the physical exercise and higher body weights on bone geometry differ, as evidenced by pQCT measurements. Finally, this study has demonstrated that food restriction in sedentary rats had a detrimental effect on the aging skeleton.

In most of the previous studies, the exercise or diet restriction program was initiated at the age of 4-5 weeks, whereas in the present study it was started at the age of 20 weeks. The reason why we started the intervention at 20 weeks of age was that we wanted to perform this experiment in rats with a more mature skeleton. It has been reported that the growth of the distal femora in male rats slows down markedly at around 5-7 months of age (SONTAG,
Male rats reach their peak bone mass at about the age of 12 months (KE et al., 2001; IIDA a. FUKUDA, 2002). The age-related decline in bone mass occurs in cancellous bone after 12 months of age, and in cortical bone after 15 months of age.

Our study has shown that voluntary running exercise can prevent the age-related osteopenia only to some extent, and that this protective effect varies among different regions within the same bone. We observed the main effect of running exercise at the tibial and femoral mid-diaphysis, much smaller effects at the distal tibia, and a lacking protective effect at the distal femur. Our findings are consistent with a previous study in adult rats showing that bone formation in response to dynamic loading varies with diaphyseal location at the ulna (HSIEH et al., 2001).

Concerning the effect of exercise on bone mass or strength, most of the studies have used treadmill exercise on a nonvoluntary basis. The results from these studies have been conflicting: Some have shown no change or decreases in mass or strength (BOURRIN et al., 1994, HOU et al., 1990), or increases in mass and strength (RAAB et al., 1990; MOSEKILDE et al., 1994; HUANG et al., 2008). These conflicting results could be due to the age of the rats, the duration and type of exercise regimes, or the skeletal sites examined.

Our treadmill exercise program at a velocity of 20 m/min for 20 minutes twice a day (i.e. 800 m/day) for five days a week showed relatively weak skeletal effects in comparison to voluntary running exercise. The possible explanation could be that the velocity and/or duration of the treadmill exercise were too low (NOWAK et al., 2008). Similar to our data, a recent study in male obese Zuker rats reported that moderate treadmill exercise failed to attenuate decreased bone growth or to promote bone mass and strength, suggesting that including some other resistance training with treadmill running may be required (IP et al., 2009). This notion is corroborated by previous experiments in young male rats which showed greater cancellous bone formation and bone mass by applying higher speed (30 m/min) and longer time intervals (60 and 90 min/day, respectively) of treadmill exercise (BOURRIN et al., 1995; JOO et al., 2003). A recent study also used an intermittent TM running protocol with higher speed (30 m/min vs. 22 m/min) to increase ground reaction force of the mild endurance exercise (HUANG et al., 2008). Interestingly, one of these study (JOO et al., 2003) reported that the adaptive response of bone to exercise differs between regions of the same bone which is also in agreement with our results.
An interesting finding in our study was that RW or TM exercise did not provide an additional benefit in terms of protection against age-related bone loss when compared with ad libitum-fed SED animals. The latter group had the highest body mass among all groups during the whole experiment. Interestingly, total BMD at the tibial distal metaphysis was higher in SED controls than in RW and TM rats at the end of the study.

The effect of body weight on BMD is well known. As weight increases, it leads to greater mechanical stress on bone and decreased bone resorption, and consequently supports the maintenance of BMD (SCHULTTHEIS, 1991; HARRIS et al., 1992). A larger body mass imposes a greater mechanical strain on bone, and in response, bone mass may increase to accommodate the greater load (RUBIN a. LANYON, 1985). Overweight has a positive influence on bone structure. Increased loading of long bones produces the greatest mechanical stresses on the subperiosteal surface and stimulates bone formation by subperiosteal expansion (FROST, 1987). A study in adult male rats with diet-induced obesity showed significantly greater bone strength in the obese rats than in the controls (BRAHMABHATT et al., 1998). The cross-sectional geometry and ultimate fracture load of the femur were higher in the obese rats than in the controls. Body weight influences both bone turnover and bone density, and is therefore a prominent modulator of vertebral and hip fracture risk, ranking in importance alongside that of age.

The positive effect of body weight on bone is probably caused by both fat mass (FM) and lean body mass (LBM), although in postmenopausal women fat mass has been more consistently demonstrated to be important. FM contributes to skeletal load in the same way as lean mass, so this simple mechanical effect may contribute to the fat-bone relationship to some extent. Moreover, FM may exert beneficial effects on bone due to increased aromatization of androgen to estrogen in adipose tissue (GREENDALE et al., 1997), lowered levels of sex hormone binding globulin (PLUIJM et al., 2001), or increased bone formation due to high circulating levels of insulin (BARRETT-CONNOR a. KRITZ-SILVERSTEIN, 1996), insulin-like growth factor 1 (BARRETT-CONNOR a. GOODMAN-GREUEN, 1998), and leptin (THOMAS a. BURGUERA, 2002). Several studies have reported FM to be an independent significant predictor of BMD among women, but not among men (REID et al., 1992; LIM et al., 2004). In contrast, MAKOVEY et al. (2005) reported a positive relationship between FM and total body BMD among men over 50, but not among their female twins.
Further studies should be undertaken to confirm the non-linear dose–response relationship of fat mass on BMD.

This study has clearly shown that neither treadmill nor running wheel exercise were able to completely prevent age-related bone loss in male rats. The data from the RW group should be considered with some caution in this respect, since the amount of the voluntary running activity decreased with age (SIPOS et al., 2008). However, femoral BMD values decreased significantly also in 23-month-old TM rats compared with 19-month-old TM rats. Therefore, forced exercise did also not completely protect against age-related bone loss.

Previous animal studies showed that adaptive bone formation can be activated by experimental loading regimes, but that the response may vary with age. The reduced skeletal response to long-term exercise treatment has been attributed to aging. However, the influence of age on bone mechano-receptiveness remains controversial (RUBIN, 1992; KLEIN-NULEND et al., 2002; PRISBY et al., 2007). There have been several reports on the responsiveness of the aged skeleton to mechanical loading. The existing experimental data have shown that the responsiveness of the aged skeleton is increased (BUHL et al., 2001), reduced (TURNER et al., 1995; RUBIN, 1992), or unaffected (RAAB et al., 1990; JARVINEN et al., 2003) compared to the younger skeleton.

One of the earlier studies demonstrated a dramatic reduction in the responsiveness of the turkey ulna to applied mechanical loads with increasing age (RUBIN, 1992). Similarly, an age-related increase in mechanical loading thresholds has been described in rats. Both the periosteal and endocortical surfaces of the tibias from 19-month-old rats were significantly less responsive to mechanical loading than those of 9-month-old rats (TURNER et al., 1995). However, other studies using cultured human bone cells found no evidence for a reduction in mechanosensitivity with donor age (KLEIN-NULEND et al., 2002). Along similar lines, a recent study reported that the bones of old male rats (75-88 weeks) displayed a clear ability to respond to a treadmill exercise that failed to initiate an adaptive response in mature animals (47-60 weeks) (LEPPÄNEN et al., 2008).

Our finding would be consistent with an age-related reduced skeletal response to loading which was reported in some earlier studies (TURNER et al., 1995; RUBIN, 1992). In addition, an age-related decrease of the bone blood flow and impairment of endothelium
dependent vasodilatation has recently been shown in male rats (PRISBY et al., 2007). Moreover, rat osteoblastic cells isolated from old animals demonstrated less basal activity of intracellular calcium and lesser response to fluid flow, relative to cells from young rats (DONAHUE et al., 2001).

Taken together, it is still unknown whether age-dependent differences in the mechanosensitivity of bone cells from young and old individuals really exist. Here, the quite liberally used terms ‘mechano-sensitivity’ and ‘mechano-responsiveness’ need to be discerned from each other. In the strictest sense, these two terms describe different phases of a multistep cellular mechanotransduction process. It is indeed possible that aging unequally affects the skeletal mechano-sensing and responsiveness, and a failure in the former could be only confirmed with direct strain measurements; i.e., similar strain surroundings would lead to lesser response among old subjects than among younger subjects. Because we did not measure bone deformations during exercise interventions, our study cannot contribute to this dilemma.

There are several possible explanations which could at least partly account for the fact that exercise did not completely prevent age-related osteopenia in this study: Firstly, the ability to produce the strain necessary to attain the bone modeling threshold may have been reduced due to decreased skeletal muscle mass and strength with age. Secondly, a decline in certain hormones or growth factors (sex steroids, parathyroid hormone, 1, 25-dihydroxy vitamin D, insulin-like growth factor (IGF), transforming growth factor β) may interact with mechanical signals to modulate the cellular mechanotransduction pathways of bone. Thirdly, age-related changes in osteoblastogenesis and osteoblast lifespan may also reduce osteocytogenesis, while increased osteocytic apoptosis may compromise osteocyte numbers which consequently leads to reduced signaling and effector response at the tissue level. Fourthly, skeletal blood flow and vascular conductance decrease with aging which may diminish interstitial fluid flow and reduce critical downstream signaling molecules such as nitric oxide (NO) within bone, which impair the adaptive remodeling machinery (PRISBY et al., 2007).

There is reason to believe that many factors contribute to the potentially reduced ability to increase bone mass through exercise with advancing age. However, neither the nature of mechanical adaptation of bone is well understood, nor the details of mechanochemical transduction within bone cells. Further investigation into the molecular mechanisms would
allow us to develop better physical exercise programs and prevent osteoporotic fractures, and may lead to potential new therapeutic targets in the treatment of metabolic bone diseases.

A remarkable finding in this study was that voluntary running exercise and increased body weight in sedentary rats affected bone properties in a different fashion. The present study has demonstrated that higher body mass in SED rats resulted in significant higher total cross-sectional area at the femoral middiaphysis compared with the RW rats. A similar effect was seen at the tibial shaft but did not reach statistical significance. Voluntary running exercise prevented expansion of the marrow cavity, so that RW rats had the lowest marrow area among the other groups of senescent animals at the middiaphyseal sites of both femur and tibia. In accordance with our results, a recent report showed that diet-induced obesity increases bone size, but reduces size-independent mechanical properties of cortical bone in mice (IONOVA-MARTIN et al., 2010).

Based on these findings, one may speculate that increased loading by voluntary exercise or overweight may have differential effects on periosteal and endocortical bone envelopes. An earlier histomorphometric study in aged female rats demonstrated that exercise affects the periosteal surface of cortical bone rather than the endosteal surface (CHEN et al., 1994). A study in tennis players suggested that the greater cortical volume in the playing compared with the non-playing arm was the result of greater endocortical contraction in the postpubertal years and greater periosteal expansion in the prepubertal years (BASS et al., 2000). The relative contributions of periosteal expansion and endocortical contraction to the larger cortical thickness in the playing than non-playing arm in another human study were 75:25 at the proximal humerus and 10:90 at both mid- and distal humerus (HAAPASALO et al., 1996). According to those findings, loading has an effect on both the periosteal and endocortical surfaces in long bones, but the intensity of the effects vary depending on location of the bone surface (anterior, posterior, medial, or lateral) and on the bone region (proximal, shaft or distal). In aging humans, it is thought that a greater periosteal apposition acts as a compensatory mechanism to offset the bone loss produced by increased endocortical remodeling during aging (SEEMAN, 2003).

In our study, the most detrimental effects on bone mass and geometry were demonstrated in the food-restricted sedentary group which showed a significant decrease in BMD and many bone structural parameters at most of the skeletal sites when compared with voluntary running
and ad libitum-fed SED animals. This is consistent with previous findings in middle-aged intact female rats, showing that dietary restriction enhanced the loss of endocortical bone with a concomitant decrease in cortical bone area and mineral content below the level for age-matched controls (BANU et al., 1999).

A limitation of our study is that in vivo fluorochrome labeling was not performed. Therefore, it was not possible to assess endocortical and periosteal bone formation rates. These parameters might have provided better insight into the effects of exercise and aging on different bone envelopes.

In conclusion, this study has shown that moderate voluntary exercise can partially, but not completely avert the age-related bone loss to a certain degree, depending on the bone site. Interestingly, the bone-protective effects of running exercise were not superior to those of increased fat mass in this experiment. The bone structural adaptation to voluntary exercise and increased body weight were different. Mild food restriction to reach paired weight with voluntarily exercising rats resulted in a clear detrimental effect on bone mass and geometry at the distal tibia and at the tibial and femoral diaphysis. Although extrapolations from rat experiments to humans always need to be treated with caution, the latter finding may also be of relevance for clinical medicine.
4. SUMMARY

The fragile and osteoporotic skeleton in old age is a growing cause of mortality and painful physical impairment in the elderly, especially in the Western world. Physical activity with dynamic loading of the bone is thought to have a beneficial effect on bone strength in children and young adults.

However, the pathophysiology of age-related bone loss, and whether age-related bone loss can be prevented by exercise are still a matter of debate. In addition, the influence of dietary intake on bone health remains largely undefined because most studies have focused their attention on calcium intake.

It was the aim of this study to investigate the long-term effects of voluntary and forced running exercise and mild food restriction on bone mineral density (BMD) and on bone geometry in the appendicular skeleton of aging male rats.

Male Sprague-Dawley rats were studied from 5 to 23 months of age. The rats were divided into 5 groups: Baseline (BL), free access to food and running wheels (RW), fed to pair weight with the RW group (PW), trained in treadmill (TM), and sedentary with free access to food (SED). All rats were housed individually. Except the baseline group, the animals were sacrificed at the age of 15, 19 or 23 months. Volumetric BMD and geometry of femurs and tibiae were assessed by peripheral quantitative computed tomography (pQCT). In addition, the tibial shafts were analyzed by cortical bone histomorphometry.

At the end of the experiment RW and PW had similar body weight. The body weight of TM and SED rats was 23% and 31% higher compared with RW rats, respectively. pQCT analysis of femurs and tibiae as well as histomorphometric analysis of the tibial shaft showed that voluntary running exercise but also higher body weight in SED rats could partially prevent the age-related bone loss. Dietary restriction resulted in lower BMD at the metaphyseal regions, and an enlargement of the marrow cavity and cortical thinning at the femoral and tibial shaft relative to the RW group. Voluntary running exercise could not completely prevent age-related bone loss between 19 and 23 months of age and also had no additional protective effects against senile osteopenia when compared to increased body weight in sedentary animals.
In summary, this thesis suggests that exercise partially protected against age-related bone loss, whereas dietary restriction had clear untoward effects on BMD and bone geometry.
5. ZUSAMMENFASSUNG

Brüchige und osteoporotische Knochen im Alter stellen eine wachsende Ursache von Sterblichkeit und schmerzhafter körperlicher Beeinträchtigung bei älteren Menschen dar, vor allem in der westlichen Welt.

Man glaubt, dass physische Aktivität mit dynamischer Belastung des Knochens eine positive Auswirkung auf die Knochenfestigkeit bei Kindern und jungen Erwachsenen hat. Die Pathophysiologie des altersbedingten Knochenverlusts und die Frage, ob altersbedingter Knochenverlust durch körperliche Aktivität verhindert werden kann, sind jedoch umstritten. Weiters ist der Einfluss der Nahrungsaufnahme auf die Knochengesundheit noch unzureichend charakterisiert, weil die meisten Studien die Aufmerksamkeit auf die Kalziumzufuhr konzentriert haben.

Es war das Ziel dieser Studie, die Langzeitwirkungen von freiwilligem und erzwungenem Laufttraining und von milder Nahrungseinschränkung auf die Knochenmineraldichte (BMD) und auf die Knochengeometrie im Extremitätskelett von männlichen Ratten während des Alterns zu untersuchen.


Am Ende des Versuches hatten RW und PW ähnliches Körpergewicht. Das Körpergewicht von TM- und SED-Ratten war jeweils 23% und 31% höher im Vergleich zu den RW-Ratten. Die pQCT-Analyse von Femur und Tibia sowie die histomorphometrische Analyse des...

Zusammenfassend lässt sich sagen, dass das Training nur teilweise gegen altersgebundenen Knochenverlust schützten konnte, während Nahrungsrestriktion klare negative Auswirkungen auf die Knochenmineraldichte und Knochengeometrie hatte.
6. REFERENCES


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7. APPENDIX

ADDITIONAL MATERIALS AND METHODS

Animal procedures

For this study, one-month-old male Sprague-Dawley rats bred under pathogen-free conditions, were obtained from the Research Institute of Laboratory Animal Breeding, Himberg, Austria (Forschungsinstitut für Versuchstierzucht und -haltung der Medizinischen Fakultät der Universität Wien). All animal procedures were approved by the Ethical Committee of the University of Veterinary Medicine Vienna and the government authorities under the animal research permit number GZ: 68.205/31-Pr/4/2002. The rats were kept at the Institute of Physiology of the University of Veterinary Medicine in Vienna, in groups of 5 animals per cage with free access to a standard rat chow at 21-23°C, and a 12h:12h light-dark cycle until the age of 5 months, when they were divided into 5 groups (n = 20 for baseline, other groups n = 64 each): (1) BL, baseline animals sacrificed at the age of 5 months, (2) RW, voluntary exercise in running wheels, (3) PW, sedentary animals fed to pair weight with the RW group, (4) TM, exercise in a treadmill, (5) SED, sedentary animals. All groups contained only rats that were willing to run in a treadmill at the age of 5 months. After start of the experiment at 5 months of age, all animals were housed individually in cages. Fifteen animals in each group were killed at 15 and 19 months, the remaining animals (n = 18 – 28 each) at 23 months of age.

The animals in the RW group had voluntary exercise in a running wheel attached to the cage. The running distance as well as the running profile (speed, activity time etc.) of the individual animals was recorded throughout the study using the electronic device that was coupled to a computer. The PW animals fed to pair weight with the RW animals received on average of 16 g food per day.
This group was designed for the comparison between the effects of achieving the same body weight by voluntary running or by strict food restriction.

The TM animals were trained in a treadmill at a velocity of 20 m/min for 20 min twice a day (i.e. 800 m/day) for five days a week. All animals had ad libitum access to water and food except the PW group. The weight of the animals was recorded monthly (groups 4 - 5) or weekly (groups 2 and 3). The animals in groups 2 - 5 were sacrificed by heart puncture under halothane anesthesia at the age of 15, 19 or 23 months. The left hind limbs were removed and stored frozen (at -20°C) until subsequent analysis.
RESULTS IN EXTENSO

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<td>562.73 ± 9.85&lt;sup&gt;ab&lt;/sup&gt;</td>
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Table 1. Changes in body weight during the experiment. Data are means ± SEM of 15 – 28 animals each. Significant differences (P<0.05) across groups at a given time point are indicated by different letters. Groups marked with the same letter are not significantly different from each other. For example, a group marked with “a” is significantly different from a group marked with “b” or “c”, but not from a group marked with “ab”. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline. SED, sedentary; PW, paired weight; RW, running wheel.
Table 2. The effects of exercise and diet restriction on total BMC, total BMD, cortical-subcortical BMD (Ctsub BMD), trabecular BMD (Trab BMD), total cross-sectional area (Total CSA), cortical-subcortical area (Ctsub Area) and trabecular area (Trab Area) of the proximal tibial metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
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**Table 3.** The effects of exercise and diet restriction on total BMC, total BMD, cortical-subcortical BMD (Ctsub BMD), trabecular BMD (Trab BMD), total cross-sectional area (Total CSA), cortical-subcortical area (Ctsub Area) and trabecular area (Trab Area) of the distal femoral metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
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**Table 4.** The effects of exercise and diet restriction on total BMC, total BMD, cortical-subcortical BMD (Ctsub BMD), trabecular BMD (Trab BMD), total cross-sectional area (Total CSA), cortical-subcortical area (Ctsub Area) and trabecular area (Trab Area) of the distal tibial metaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
<table>
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Table 5. The effects of exercise and diet restriction on total BMC, total BMD, cortical BMD (Ct BMD), cortical thickness (Ct Thickness), total cross-sectional area (Total CSA), cortical area (Ct Area), and marrow area of the tibial shaft assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
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**Table 6.** The effects of exercise and diet restriction on total BMC, total BMD, cortical BMD (Ct BMD), cortical thickness (Ct Thickness), total cross-sectional area (Total CSA), cortical area (Ct Area), and marrow area of the femoral mid-diaphysis assessed by pQCT. Data are means ± SEM of 15 – 28 animals each. Significant differences across groups at a given time point are indicated by different letters. +, P<0.05 vs. 23-month-old rats (only shown for 19-month time point). *, P<0.05 vs. baseline.
Table 7. Histomorphometric analysis of microground cross-sections of the tibial shaft from baseline and 23-month-old rats. Representative cross-sections of the tibial shaft are shown. The enlarged marrow cavity in PW relative to RW rats is clearly evident. Fine-ground, 20-µm-thick sections. Toluidine blue stain. Total cross-sectional area (Total CSA), cortical area (Ct Area), percent cortical area (percent Ct Area), marrow area, percent marrow area and cortical thickness (Ct Thickness) of the tibial shaft measured by histomorphometry. Data are means ± SEM of 18–28 animals each. Significant differences (P<0.05) across groups are indicated by different letters. *, P<0.05 vs. baseline.

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<th>PW</th>
<th>TM</th>
<th>SED</th>
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<td>23 7.06 ± 0.096ᵃ</td>
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<td>Ct Area (mm²)</td>
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<td>23 5.24 ± 0.070ᵃᵇ</td>
<td>5 4.98 ± 0.084ᵃᵇ</td>
<td>23 5.18 ± 0.081ᵃᵇ</td>
<td>5 5.46 ± 0.076ᵃᵇ</td>
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<td>Percent Ct Area (%)</td>
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<td>23 74.41 ± 0.94ᵇ</td>
<td>5 70.01 ± 0.89ᶜ</td>
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<td>5 73.73 ± 0.99ᵇᶜ</td>
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<td>Marrow Area (mm²)</td>
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<td>Percent Marrow Area (%)</td>
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<td>Ct Thickness (mm)</td>
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<td>5 0.722 ± 0.011ᵃᵈ</td>
<td>23 0.755 ± 0.014ᵃᵈ</td>
<td>5 0.798 ± 0.015ᵃᵇᶜ</td>
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